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(54) Title: GENETICALLY MODIFIED PLANTS TOLERANT OF STRESS CONDITIONS

(57) Abstract

The present invention provides a genetically modified plant having improved stress tolerance, which plant expresses a recombinant homoserine acetyltransferase (HAT). The present invention further provides constructs and methods for generating the stress tolerant plant.

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GENETICALLY MODIFIED PLANTS TOLERANT OF STRESS CONDITIONS

FIELD OF THE INVENTION

The present invention relates to genetically modified plants having improved stress tolerance and to nucleic acid constructs useful in the generation of such genetically modified plants. The present invention further relates to the use of a modified amino acid and it's derivatives for conferring stress tolerance in wild type plants.

ABBREVIATIONS

AK: aspartate kinase; **AHS:** acetyl homoserine sulfhydrylase; **CL:** cystathionine β-lyase; **CS:** cystathionine γ-synthase; **DHPS**: dihydrodipicolinate synthase; **HAT:** homoserine acetyltransferase; **HK:** homoserine kinase; **HSD:** homoserine dehydrogenase; **MS:** methionine synthase; **PHS:** *O*-phosphohomoserine; **SAM:** *S*-adenosyl methionine; **SAMS:** SAM synthase. **TDH:** threonine dehydratase; **TS:** threonine synthase.

BACKGROUND OF THE INVENTION

Biotic and abiotic stresses cause considerable losses in crop quality and productivity. Genetic improvement of stress tolerance is therefore an urgent need. There are two basic strategies to engineer stress tolerance in plants, as follows: (i) plant mutagenesis; and (ii) transformation of plants with specific genes conferring tolerance. The selected genes may be derived from several sources such as animal, yeast or bacteria, and possibly implement metabolites normally not synthesized in plants. The selected genes may represent key enzymes of identified pathways which lead to the accumulation of desirable metabolites when manipulated [Bartels and Nelson (1994) Plant cell Environment 17:659-667].

A common effect of many environmental stresses is to cause oxidative damage. One of the important mechanisms by which plants are damaged

during adverse environmental conditions is the excess production of reactive oxygen species (ROS), such as superoxide and hydroxyl radicals. Such oxidative stress has been shown to occur in plants exposed to high and low temperature, high light intensities, drought, high salinity, exposure to air pollutants, U.V. light and herbicides such as paraquat. A large increase in ROS is observed rapidly upon infection of plants with pathogens or upon elicitor treatment. This oxidative burst is thought to be responsible for hypersensitive cell death, as well as the rapid cross-linking of hydroxyproline-rich glycoproteins in the cell wall, associated with active pathogen defense. ROS might also serve as secondary messengers responsible for activating pathogenesis-related genes and genes involved in phytoalexin biosynthesis.

A strong correlation was found between resistance to paraquat (a herbicide causes oxidative stress) and resistance against other abiotic stresses, such as drought, heat, salinity and cold. Paraquat causes oxidative stress by competing for the electron flow in the electron transport chain during photosynthesis. This results in the production of the radical superoxide. Superoxide can act either as a potent oxidant, forming hydroxyl radicals, or as a reductant, forming singlet oxygen, both are ROS, causing chlorophyll bleaching and severe damage to the membrane. Plant cells can be protected against oxidant stress by various radical-scavenging mechanisms, involving antioxidants of low molecular weight, or antioxidant enzymes [Trebst (1991) In Herbicides Resistance in weeds and crops, Caseley, JC. Cussans, GW., Eds Butterworths Oxford, 145].

Plants use also several other mechanisms to defend against, or to adapt to, different stresses. The water deficiency resulting from salt stress has been well studied. Adaptation to water deficiency can involve the accumulation of osmolytes, such as proline [Igarashi et al. (1997) Plant Mol. Biol. 33:857-865]. Plants protect themselves against ultra-violet-B light (UV-B - 280-320 nm) by producing phenol flavonoid compounds, which accumulate in the

epidermal cells and absorb the UV light. It was found that UV-B causes oxidative stress and that phenolic compounds can be activated by the absorption of UV-B light.

In addition, when a plant recognizes a pathogen attack, it responds by inducing several local responses in the cells immediately surrounding the infected site. These include a localized cell death, known as hypersensitive response, characterized by the deposition of callose, physical thickening of cell walls by lignification, as well as the synthesis of various small antibiotic molecules, such as phytoalexins. Genetic factors in both the host and the pathogen determined the specificity of these local responses, which can be very effective in limiting the spread of infection.

The present invention provides polynucleotide constructs and methods utilizing same for conferring improved stress tolerance to plants.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided an isolated polynucleotide comprising: (a) a first nucleic acid sequence encoding an enzyme having homoserine acetyltransferase (HAT) activity or a second nucleic acid sequence encoding an enzyme having acetylhomoserine sulfhydrylase (AHS) activity; and (b) a third nucleic acid sequences capable of enabling the expression of HAT or AHS in plant cells and, optionally, the transportation of the expressed HAT or AHS to the plastids.

According to further features in preferred embodiments of the invention described below, the isolated polynucleotide comprising both the first and the second nucleic acid sequences.

According to still further features in the described preferred embodiments the third nucleic acid sequence includes: (i) a plant promoter; and (ii) a plant polyadenylation and termination sequence.

According to still further features in the described preferred embodiments the third nucleic acid sequence includes: (i) a plant promoter; (ii) a plant polyadenylation and termination sequence; and (iii) a sequence encoding a plastid transit peptide translationally fused to the 5'-end of the first or the second nucleic acid sequence for the transportation of the expressed HAT or AHS to the plastids.

According to still further features in the described preferred embodiments the plant promoter is selected from the group consisting of a constitutive promoter, a tissue specific promoter, an inducible promoter and a chimeric promoter.

According to still further features in the described preferred embodiments the constitutive plant promoter is selected from the group consisting of CaMV 35S promoter, CaMV19S promoter, FMV34S promoter, sugarcane bacilliform badnavirus promoter, CsVMV promoter, *Arabidopsis* ACT2/ACT8 actin promoter, *Arabidopsis* ubiquitin UBQ1 promoter, barley leaf thionin BTH6 promoter, and rice actin promoter.

According to still further features in the described preferred embodiments the tissue specific plant promoter is selected from the group consisting of bean phaseolin storage protein promoter, DLEC promoter, PHSβ promoter, zein storage protein promoter, conglutin gamma promoter from soybean, AT2S1 gene promoter, ACT11 actin promoter from *Arabidopsis,* napA promoter from *Brassica napus* and potato patatin gene promoter.

According to still further features in the described preferred embodiments the inducible promoter is selected from the group consisting of a promoter induced in stress conditions comprising light, temperature, drought, high salinity, osmotic shock, oxidant, chemical or pathogenic stress, being selected from the light-inducible promoter derived from the pea rbcS gene, the promoter from the alfalfa rbcS gene, the promoters DRE, MYC and MYB active in drought; the promoters INT, INPS, prxEa, Ha hsp17.7G4 and RD21

active in high salinity and osmotic stress, the promoters hsr303J and str246C active in pathogenic stress; the rd29A promoter active in dehydration induced by drought, salt loading and by freezing; the stilbene promoter active during UV radiation and high level of ozone, and the promoters hsr303J and str246C active in pathogenic stress.

According to still further features in the described preferred embodiments the first or the second nucleic acid sequence is derived from yeast or from bacteria, or is a synthetic gene.

According to still further features in the described preferred embodiments the first nucleic acid sequence includes a sequence as set forth in SEQ ID NO:5 or a functional portion thereof.

According to still further features in the described preferred embodiments the second nucleic acid sequence includes a sequence as set forth in SEQ ID NO:6 or a functional portion thereof.

According to still further features in the described preferred embodiments there is provided an expression vector comprising the isolated polynucleotide described above.

According to still further features in the described preferred embodiments there is provided a transgenic plant, plant derived tissue or a plant cell comprising the isolated polynucleotide described above.

According to still further features in the described preferred embodiments there is provided a genetically modified plant comprising the isolated polynucleotide described above, having an improved tolerance to a stress condition as compared with a corresponding wild-type plant, the stress condition is selected from the group consisting of high salinity stress, drought stress, temperature stress, mineral deficiency stress, osmotic stress, oxidant stress, chemical stress and pathogenic stress.

According to another aspect of the present invention there is provided a genetically modified plant comprising the met2 gene of *Saccharomyces cerevisiae* or the metX gene of *Leptospira meyer*.

According to still another aspect of the present invention there is provided a genetically modified plant having an increased free content, as compared with a corresponding wild-type plant, of arginine and ornithine and/or its related metabolites selected from the group consisting of citrulline, putrescine, nitric oxide, spermine and/or spermidine.

According to yet another aspect of the present invention there is provided a genetically modified plant having an increased free content, as compared with a corresponding wild-type plant, of phenols and/or phenols related metabolites.

According to an additional aspect of the present invention there is provided a method for producing a genetically modified plant having an increased free content, as compared with a corresponding wild-type plant, of arginine and ornithine and/or its related metabolites, the method comprising the steps of: (a) transforming plant cells with an isolated polynucleotide capable of expressing in plants, an enzyme having homoserine acetyltransferase (HAT) activity or an enzyme having acetylhomoserine sulfhydrylase activity or both; and (b) regenerating transformed plants from the transformed plant cells of (a) and selecting for plants that express HAT.

According to yet an additional aspect of the present invention there is provided a method for producing a genetically modified plant having an improved tolerance to a stress condition as compared with a corresponding wild-type plant, the method comprising the steps of: (a) transforming plant cells with an isolated polynucleotide capable of expressing in plants, an enzyme having homoserine acetyltransferase (HAT) activity; and (b) regenerating transformed plants from the transformed plant cells of (a) and

selecting for plants having an improved tolerance to a stress condition as compared with a corresponding wild-type plant.

According to still further features in the described preferred embodiments the enzyme having homoserine acetyltransferase (HAT) activity is selected from the group consisting of yeast met2 and bacterial metX.

According to still further features in the described preferred embodiments the stress condition is selected from the group consisting of high salinity stress, drought stress, temperature stress, mineral deficiency stress, osmotic stress, oxidant stress, chemical stress and pathogenic stress.

According to still further features in the described preferred embodiments the step of selecting (step c) is effected by growing the transformed plants under the stress conditions.

According to still a further aspect of the present invention there is provided a method of increasing the stress tolerance of a plant, the method comprising the step of applying to the plant homoserine or homoserine derivatives.

According to still further features in the described preferred embodiments the homoserine or homoserine derivatives are externally applied to the plant.

According to still further features in the described preferred embodiments the homoserine or the homoserine derivatives are provided in a liquid solution.

According to still further features in the described preferred embodiments the liquid solution also includes a surfactant.

According to still a further aspect of the present invention there is provided a plant fertilizer comprising a fertilizer base and homoserine or homoserine derivatives, wherein the homoserine or the homoserine derivatives are provided in a concentration sufficient to induce stress tolerance within a plant growing on the plant fertilizer.

The present invention successfully addresses the shortcomings of the presently known configurations by providing a transgenic plant tolerant of a wide range of environmental and pathogen related stress conditions.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIG. 1 is a diagram of the aspartate-family biosynthetic pathway in plants. Major regulatory enzymes and their products are indicated. Feedback inhibition (-) and activation (+) loops are shown by dashed-line arrows.

FIG. 2a is a diagram of the plant (solid lines) and yeast, fungi and bacteria (dashed-line) pathways for methionine biosynthesis.

FIG. 2b is a diagram of fluxes associated with methionine metabolism in plants. Abbreviations: AdoMet- S-adenosylmethionine; AdoHcy-S-adenosylhomocysteine; MTA- methylthio adenosine; SMM- S-methyl methionine; DMSP- dimethylsulfoniopropionate.

FIG. 3 is a diagram of the arginine and proline biosynthesis pathways. The intermediates are indicated by their abbreviations: γ-GP, γ-glutamyl phosphate; GSA, γ-glutamyl- 5-semialdehyde; P5C, pyrroline-5- carboxylate;

KVA, α-keto-δ-aminovalerate; P2C, pyrroline-2-carboxylate; AcG, *N*-acetylglutamate; AcGP, AcG-5-phosphate; AcGSA, AcG-5-semialdehyde; AcORN, *N*-acetylornithine; CP, carbamoyl- phosphate; CIT, citrulline; ASP, aspartate; AS, arginosuccinate; AGM, agmatine; NCPUT, *N*-carbamoylputrescine. The thinner arrows indicate the lower possibility in the pathway.

The enzymes are indicated by numbers within brackets: (1) γ-Glutamyl kinase; (2) GP reductase; (2') P5C synthase; (3) non-enzymatic reaction: GSA → P5C; (4) P5C reductase; (5) δ-ornithine aminotransferase; (6) P5C reductase; (7) Ornithine α-aminotransferase; (8) non-enzymatic reaction: KVA→ P2C; (9) P2C reductase; (10) proline dehydrogenase; (11) P5C dehydrogenase; (12) AcG synthase; (13) *N*-acetyltransferase; (14) AcG kinase; (15) AcGSA oxidoreductase; (16) AcORN aminotransferase; (17) AcORN decarboxylase; (18) ornithine carbamoyltransferase; (19) argininosuccinate synthase; (20) argininosuccinate lyase; (21) arginase; (22) urease; (23) arginine decarboxylase; (24) AGM iminohydrolase; (25) NCPUT amidohydrolase; (26) ornithine decarboxylase.

FIGs. 4a-d are schematic diagrams of nucleic acid constructs (cassettes) used according to the present invention for expression of each of the *Saccharomyces cerevisiae* met2 and met25 genes encoding HAT and AHS, respectively, or both and targeting of the expressed proteins to the cytosol (Figures 4a and 4c) and to the plastids (Figures 4b and 4d) metX gene encoding HAT targeting the expressed protein to the cytosol or to the plastid (Figures 4a or 4b). The chimeric genes are shown in their position between left (LB) and right (RB) borders of the binary vector T-DNA [pGPTV-HPT-105 carrying the gene for hygromycin resistance (hpt). Abbreviations: PRO, promoter (CaMV35SΩ - constitutive CaMV35S promoter linked to the Ω translation enhancer sequence) or the bean phaseolin storage protein promoter for seed-specific expression); TER, terminator; TP, transit peptide targeting

the enzyme to plastids; Pnos, promoter of nopaline synthase gene of *Agrobacterium tumefaciens*; pAg7, gene 7 poly (A) of *Agrobacterium tumefaciens*.

FIGs. 5a-f show the coding nucleotide sequences of the *Saccharomyces cerevisiae* met2, met25 and of the bacterial *Leptospira meyeri* genes (Figures 5b, 5d and 5f, respectively) used in the constructs of Figure 4, and the amino acid sequences of the proteins HAT and AHS encoded thereby (Figures 5a, 5e and 5c, respectively).

FIGs. 6-7 are schematic diagrams of nucleic acid constructs (cassettes) for expression of the *E. coli lysC* gene encoding a mutant aspartate kinase (Figure 6) and of the 10 Kd zein gene (Figure 7) in plants.

FIGs. 8a-b show Northern blot analysis of representative homozygous transgenic tobacco plants expressing the yeast met2 gene (Figure 8a) and the expressing of the bacterial metX (Figure 8b) under the control of the CaMV35SΩ promoter.

FIGs. 9a-b show Western protein dot blots of HAT expressed in transgenic tobacco plants by the yeast met2 gene under control of the CaMV35SΩ promoter (Figure 9a) and under control of the seed-specific bean phaseolin storage protein promoter (Figure 9b). Proteins extracted from leaves (Figure 9a) or seeds (Figure 9b) of the transgenic plants or of wild-type plants (control) were reacted with antibodies specific to HAT. Abbreviations: P. Cont., positive control from the glutathione S-transferase-HAT fusion protein; N. Cont., negative control from extracts from wild-type plants.

FIGs. 10a-b show Western blots showing chloroplastic and cytosolic (cytoplasmic) compartmentalization of AHS expressed in transgenic tobacco plants by the yeast met25 gene under control of the constitutive CaMV35SΩ promoter (Figure 10a) and under control of the seed-specific bean phaseolin storage protein promoter (Figure 10b). Proteins extracted from leaves (Figure 10a) or seeds (Figure 10b) of the transgenic plants containing the cytoplasmic

and chloroplastic-type AHS enzymes or of wild-type plants (control) were reacted with antibodies specific to AHS.

FIGs. 11a-b show Western protein dot blots of the progenies of the crossing between the plants expressing the yeast met2 gene (encoding HAT) and plants expressing the yeast met25 gene (encoding AHS) under control of the constitutive CaMV35SΩ promoter, using anti-HAT antibodies (Figure 11a) and anti-AHS antibodies (Figure 11b). The crosses are: 1, 2-205 x 17; 3, 4-110 x 17; 5, 6-17 x 205; 7, 8-17 x 110 (female plants are indicated on the left).

FIGs. 12a-b show the total phenols, of homozygous transgenic plants expressing the met2 gene, and untransformed plants (WT) (Figure 12a) and transgenic plants expressing the metX gene (Figure 12b) as measured according to quercitin standard. Bars represent SD of mean, the averages are from five plants.

FIG. 13 show the chlorophyll levels in transgenic plants and untransformed plants (WT) after 4 h in 10^{-5} mM paraquat solution. The Figure allows comparison between the chlorophyll level of disks placed in paraquat and control disks placed in water. Bars represent SD of mean, the averages are from seven plates.

FIGs. 14a-b show the effect of TMV infection on leaves of homozygous plants expressing the yeast met2 gene and untransformed plants (WT). Figure 14a shows the number of TMV lesions formed on the leaves of plants four days after infection (average from six plants). Figure 14b shows representative leaves presenting the TMV lesions from untransformed plants, 17 and 12. The number and lesion diameter can distinguish.

FIG. 15 show the total phenols in leaves 4 and 5 in transgenic line 17 and untransformed plants (WT) after radiation with UV-B. The samples were removed after 3, 6 and 12 h. Bars represent SD of mean, the averages are from four plants.

FIG. 16 show the osmotic adjustment of lines 17 and untransformed plants (WT) under drought stress. The control is irrigated plants. Bars represent SD of mean, the averages are from five plants.

FIG. 17 show the total phenols in cut off leaves after 48 h in various solutions at the greenhouse. Abbreviation: AHS-acetyl homoserine; HOL-homoserine lactone; HOM-homoserine; GLN-glutamine; ASN- aspargine; DDW-double distilled water.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of genetically modified plants having improved stress tolerance and to nucleic acid constructs useful in the generation of such genetically modified plants. The present invention is further of the use of a modified amino acid and it's derivatives for conferring stress tolerance in wild type plants.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the present invention is not limiting its application to the details of construction and components set forth in the following description or illustrated in the drawings or examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

In a pursue for genetically modified plants overexpressing methionine, the inventors of the present invention have unexpectedly realized that plants overexpressing homoserine acetyltransferase (HAT) are over producing arginine and are more tolerant to various stress conditions as is compared to wild type plants. The following descriptions provide (i) the rational for

producing plants expressing high levels of methionine; (ii) background information of methionine metabolism and catabolism; (iii) background information of arginine metabolism and catabolism; and (iv) disclosure of the present invention. These descriptions are followed by an Examples section which describe in more detail the reduction of the present invention to practice.

Methionine consumption:

The diet of humans and livestock is largely or solely based on plant material in the form of food or feed. However, crop plants are generally considered to be a low nutritional quality food source because they contain low content of several amino acids which are essential for, but cannot be synthesized by, monogastric animals. Therefore, synthetic or bacterial-produced essential amino acids are usually supplied as supplements to grain-based and other diets, in order to increase their essential amino acid content and thus improve their nutritional value.

One of the most important essential amino acid is methionine, which is also the scarcest essential amino acid in, for example, legumes and other crops. Therefore, legume-based animal diets are generally supplemented with chemically-produced free methionine. In 1998 alone, the world consumption of such free methionine as a soy-based feed supplement was 400,000 tons. However, synthetic methionine is considered a low quality food/feed product because its production requires the use of strong organic solvents and residues thereof remain in the final product. There is thus a long felt need for nutritionally balanced crop plants that produce larger amounts of methionine.

Various attempts have been made in the past to raise the methionine content in plants by breeding and selection. However, these attempts failed to provide methionine enriched agronomically-acceptable cultivars [see, for example, Madison and Thompson, 1988].

There is thus a widely recognized need for, and it would be highly advantageous to have plants overproducing and accumulating methionine, because such plants have an improved nutritional value for human and livestock consumption.

One genetic engineering approach to increase the methionine content in seeds and vegetative tissues of plants is to link a gene or genes encoding for proteins that are inherently rich in, or that are enriched to include methionine, to strong constitutive or tissue-specific regulatory sequences, to transform crop plants therewith and to identify transformants in which the gene or genes are sufficiently highly expressed to cause a pronounced increase in total methionine content. This approach is limited, however, by the ability of the transformed plants to synthesize methionine. In such a case, the internal competition for free methionine may lead to underproduction of other proteins which include methionine and which are essential for plant flourishing or survival. This, in turn, defines an upper limit to the ability to exploit the free methionine sources of the plant by the proposed approach.

An alternative approach would be to increase the production of free methionine via genetic engineering techniques by intervention in the methionine synthesis pathway. However, little is known of the control of the biosynthesis and metabolism of methionine in plants, particularly in plant seeds.

Methionine biosynthesis in plants:

The essential amino acids lysine, threonine and methionine are synthesized from aspartate by a complex pathway that is similar in bacteria and in higher plants. The biosynthesis of the aspartate family comprising the amino acids lysine, threonine, methionine and isoleucine is depicted in Figures 1 and 2. The carbon skeleton of methionine is derived from aspartate, the sulfur atom from cysteine and the methyl group from *N*-methyltetrahydrofolate, a triglutamyl derivative (Bryan, 1980).

As shown in Figures 1-2a-b, the methionine pathway diverges from the threonine branch of the aspartate-family synthesis pathway following the generation of *O*-phosphohomoserine (PHS). The first enzyme specific for methionine biosynthesis in plants, cystathionine γ-synthase (CS), catalyzes a central step in methionine synthesis by joining metabolites from two separate pathways: PHS (from the aspartate family pathway) and cysteine (from the cysteine biosynthesis pathway), to form cystathionine, that is then converted to homocysteine by the trans-sulfurylase cystathionine β-lyase (CL). In addition to trans-sulfurylation, an alternative pathway for homocysteine biosynthesis is the direct sulfhydration pathway, in which sulfide substitutes for cysteine as a source of sulfur atom to form homocysteine, but this pathway has been established as of little, if any, physiological significance in the synthesis of methionine in plants (Datko, 1977). The final step in methionine biosynthesis in plants is catalyzed by methionine synthase (MS) which converts homocysteine to methionine.

The aspartate-family biosynthetic pathway involves many enzymes, some of which are common for several or all of the aspartate family amino acids, while others are specific for individual amino acids. The rate of synthesis of the amino acids is regulated by a complex process of inhibition of the activity of some key enzymes in the pathway by the relevant amino acid end product(s). The major rate-limiting enzyme of the threonine/methionine branch pathway is aspartate kinase (AK), also known as aspartokinase. AK controls the production of all the aspartate-family amino acids, and is subject to feedback-inhibition by lysine and/or threonine, and also by the methionine metabolite, *S*-adenosyl methionine (SAM) which is produced from methionine by SAM synthase (SAMS, Figure 1).

The key role of aspartate kinase in regulating the threonine pathway has been demonstrated by expressing a feedback inhibition insensitive bacterial aspartate kinase, either constitutively or in a seed-specific manner, in

transgenic tobacco plants (Galili, 1995; EP 0485970, which are incorporated by reference as fully set forth herein). This resulted in a significant overproduction of threonine but, surprisingly, only in a small increase in methionine (Shaul *et al*, 1992). This fact suggests that CS competes inefficiently with threonine synthase (TS) for their common substrate PHS. This hypothesis was supported by the finding that threonine synthase has a much lower Km for PHS than CS (Thompson, 1982).

These and other biochemical studies suggest that the competition between the methionine and threonine pathways on the carbon skeleton is crucial for methionine biosynthesis in plants. However, this is not the case in other organisms, such as bacteria, yeast and fungi, which possess alternative pathways for methionine biosynthesis than those of plants, thereby bypassing the regulatory point at CS.

The methionine biosynthesis pathway and the regulation thereof are interesting not only for the purpose of man intervention directed at elevating the methionine content, but also from an evolutionary point of view. Understanding the methionine biosynthesis pathway has contributed to the development of herbicides that inhibit the methionine biosynthesis enzymes (Azevedo *et al.*, 1997). New herbicide designers are constantly seeking for biosynthesis pathways which are operative in plants and are absent in animals, so as to develop safer herbicides. The biosynthesis pathway of methionine is a good target since methionine is an essential amino acid for animals.

Methionine biosynthesis in yeast, fungi and bacteria:

In yeast, fungi and bacteria the methionine biosynthesis pathway diverges from the aspartate homoserine, one intermediate metabolite upstream to PHS on the threonine biosynthesis pathway of plants. As shown in Figure 2a, dashed lines, homoserine is combined with acetyl coenzyme A to form acetylhomoserine. This reaction is catalyzed by homoserine acetyltransferase (HAT) which is encoded, for example, by the met2 gene of the yeast

Saccharomyces cerevisiae. Next, acetylhomoserine is converted into homocysteine, a reaction catalyzed by acetylhomoserine sulfhydrylase (AHS) which is encoded, for example, by the met25 gene of *Saccharomyces cerevisiae*. AHS synthesizes homocysteine and cysteine through sulfhydrylation with sulfide of acetylhomoserine and acetylserine, respectively. AHS has been thus characterized as a bifunctional sulfhydrylase (Antoniewski *et al.*, 1973). Homocysteine serves as a substrate for the production of both methionine and cysteine, indicating that cysteine is formed through acetylserine sulfhydrylase, as well as through reverse trans-sulfurylation (Cherest, 1993). The met2 and met25 genes, as well as other genes in the methionine biosynthesis pathway in *Saccharomyces cerevisiae,* are regulated by the repression of methionine and SAM (Cherest *et al.*, 1971).

Thus, the methionine synthesis pathway in yeast differs from the main methionine synthesis pathway in plants in two major aspects: (i) the methionine and threonine branches compete for the common intermediate homoserine, rather than for PHS; and (ii) the main pathway for homocysteine biosynthesis in yeast is catalyzed by AHS, using sulfide as a source of sulfur, rather than via the trans-sulfurylation pathway catalyzed by CS and CL, that uses cysteine as a source of sulfur.

Arginine biosynthesis and function in plants:

The synthesis of arginine has been studied only scarcely in plants (Verma, 1998). Biochemical studies suggest that the arginine biosynthesis pathway in plants is similar to that operating in bacteria. As shown in Figure 3, a major intermediate product in the pathway of arginine biosynthesis is ornithine, and both arginine and ornithine serve as donors for production of the polyamine putrescine. Biochemical studies suggest that a major limiting factor for arginine biosynthesis occurs at the level of the first enzyme in the pathway, namely, N-acetylglutamate synthase [enzyme (12) in Figure 3], which combines glutamate and acetyl CoA to produce N-acetylglutamate (AcG). This

enzyme is subjected to end product feedback inhibition by arginine. Intracellular localization studies also suggest that most, if not all, of the enzymes of arginine biosynthesis in plants are localized in the plastids, similar to the enzymes associated with other amino acid pathways.

Beside its role as a building block of proteins, arginine serves additional functions in plants. Together with its intermediate compound ornithine, arginine serves as donor of the polyamine putrescine, which is further converted into the polyamines spermidine and spermine. These polyamines play important roles in plant development, cellular proliferation and normal cell function and are also involved in the response of plants to salt and osmotic stresses (Chattopadhyay *et al.*, 1997). Putrescine levels are known to accumulate in response to various abiotic stresses like K+ deficiency (Smith, 1973), acid stress (Young *et al.*, 1983), oxidant stress (Ye *et al.*, 1997), and osmotic stress (Flores *et al.*, 1982). Moreover, expression of the genes encoding arginine decarboxylase and ornithine decarboxylase, two enzymes which participate in the biosynthesis of putrescine, were shown to be stimulated by salt and osmotic stresses (Chattopadhyay *et al.*, 1997). In addition, the expression level of genes encoding for these enzymes was shown to be positively correlated with salt resistance in various plant species including different rice cultivars (Basu *et al.*, 1991).

In plants, the arginine biosynthesis pathway further provides the precursor ornithine, in particular, for the synthesis of the important amino acid proline, which accumulation has been correlated, in a variety of organisms, to adaptation to osmotic stresses (Delaney and Verma, 1993). Proline accumulation in plants under stress conditions also functions in the processes of energy, amino nitrogen and reducing power storage. The conversion of ornithine to proline can occur through two alternative routes, both involving transamination of ornithine, the main route being through pyrroline-5-carboxylate (P5C). Application of radioactive ornithine or arginine to

organisms results in the formation of radioactively-labeled proline. In plants, this labeling is enhanced by water stress [Bogess *et al.*, 1976). Moreover, calculations based on the specific activity of the precursors, the pool sizes of free arginine and free glutamate, and the transfer rates of radioactivity to proline, indicate that arginine may be more important than glutamate as a precursor for the synthesis of proline during water deficiency stress (Stewart and Bogess, 1977).

Thus, taken together, during stress, arginine may play a pivotal role in the synthesis of both proline and putrescine, two important metabolites which accumulate during stress and protect the plant. The arginine biosynthesis pathway was found to be activated during salinity stress, resulting in arginine accumulation at levels 2-3 fold higher as compared with control plants. The ability to elevate arginine during salinity stress was found to be directly correlated to the resistance of different plant species to salinity stress (Lazcano-Ferrat and Lovatt, 1997).

The accumulation of the arginine pathway intermediates, ornithine and citrulline (CIT), is also associated in a variety of higher plant species with a number of mineral nutrient deficiencies: phosphate, ferrum, zinc and also in deficiencies of magnesium, sulfur, manganese, chloride, copper and potassium (Rabe and Lovatt, 1984; Rabe and Lovatt, 1986, and references therein). Rabe and Lovatt (1986) suggested that during mineral deficiencies, nitrate and ammonia accumulate and are subsequently removed through *de novo* arginine biosynthesis. Arginine has four nitrogen atoms, thus, *de novo* arginine biosynthesis provides an efficient mechanism for detoxification of excess tissue ammonia.

Arginine also serves as a precursor for the biosynthesis of nitric oxide (NO), known to be involved in various processes in mammalian cells and recently shown to be involved in the response of plant cells against pathogen attacks [Durner *et al.*, 1998; Hausladen *et al.*, 1998). It was found, in this

respect, that infection of resistant, but not susceptible, tobacco plants with tobacco mosaic virus resulted in enhanced NO synthase activity [Durner *et al.*, 1998).

Arginine also serves as an efficient nitrogen storage means in seeds. This is because arginine contains four nitrogen atoms per molecule and the release of nitrogen upon arginine degradation supports the growth of seedlings during germination. A similar degradation of arginine during vegetative senescence supports the release of nitrogen transported into the seeds to support seed development.

While reducing the present invention to practice and in an effort to produce plants which overproduce and accumulate methionine, unexpectedly, it was uncovered that the yeast met2 gene and more so, the bacterial metX gene, when expressed in transgenic plants, function in conferring stress tolerance to such plants. Plants transformed with these genes, both encode for homoserine acetyltransferase (HAT) possess elevated levels of arginine and phenols, compounds which confer such stress tolerance. Phenols protect plants against stress in a number of ways, for example, by serving as antioxidants. They are also precursors to lignin (a component of the cell wall), which protects the plant against pathogen invasion. A number of phenols are phytoalexins, which act as antibiotic agents against various pathogens.

Evidently, there is a widely recognized need for, and it would be highly advantageous to have plants overproducing and accumulating arginine and phenols because such plants will have the ability to (i) defend against pathogen attack; (ii) to respond to salt, oxidant, drought and osmotic stresses, as well as other stresses. In addition, such plants will have the ability to (iii) synthesize higher levels of polyamines which play important roles in plant development, cellular proliferation and normal cell function, as well as in plant response to salt, drought and osmotic stresses and other abiotic stresses like K^+ deficiency, oxidant stress and osmotic stress; (iv) synthesize higher levels of nitric oxide

(NO) which is involved in the response of plant cells against pathogen attacks; (v) produce higher nitrogen storage pools which are required for efficient germination of seedlings and for vegetative senescence; and (vi) produce high levels of proline under osmotic stress and exhibiting higher tolerance to mineral deficiency.

According to one aspect of the present invention there is provided a genetically modified plant expressing a recombinant homoserine acetyltransferase (HAT). The nucleic acid sequence encoding the recombinant HAT can be of any non-plant origin, e.g. from yeast fungi or bacteria, or may be a synthetic gene. Preferably, it is encoded by, for example, the met2 gene of the yeast *Saccharomyces cerevisiae* [ENTREZ AJ001940, M15675; Langin *et al.*, 1986], the metX gene from the bacterium *Leptospira meyeri* [ENTREZ Y10744, Bourhy *et al.*, 1997], the met2 gene of the filamentous ascomycetes *Ascobolus immersus* [ENTREZ M26662, Goyon *et al.*, 1989], a cDNA from *Acremonium chrysogenum* [AN E08839, Japanese Patent Application No. JP 1995067649-A1], the metA gene of the bacterium *Corynebacterium glutamicum* [ENTREZ AF052652] or the gene from operon gerA of *Bacillus cereus* [ENTREZ AF067645].

The genetically modified plants of the invention expressing the HAT encoded by the *S. cerevisiae* met2 gene or by *L. meyeri* metX were found to contain an increased free content of arginine and ornithine as compared with corresponding wild-type plants. However, the levels of methionine in these plants as compared to wild type plants, remained unchanged although it is expected that these plants also contain an increased free content, as compared with a corresponding wild-type plant, of other arginine related metabolites such as citrulline, putrescine, nitric oxide, spermine and/or spermidine.

It will be appreciated that although the transgenic plants of the present invention failed to provide the results expected, more importantly, and surprisingly the genetically modified plants of the present invention expressing

the HAT encoded by the *S. cerevisiae* met2 gene or by *L. meyeri* metX gene were found to contain an increased content of total phenols as compared with corresponding wild-type plants. It is expected that these plants also contain an increased free content, as compared with a corresponding wild-type plant, of other phenols related metabolites such as flavanoides (serving as antioxidants), precursors for lignin, and phytoalexins. Further detail of the above results is given in Example 7 of the Examples section which follows. As a result of the high levels of phenols accumulated therein, the genetically modified plants of the invention expressing the HAT encoded by the *S. cerevisiae* met2 coding sequence or the *L. meyeri* metX coding sequence were found to tolerate salt stress, drought, TMV infection, cold, U.V. radiation and oxidant stress conditions and it is expected that they will have an improved tolerance to other stress conditions as compared with corresponding wild-type plants, such as , but not limited to, drought stress, temperature stress, mineral deficiency stress, osmotic stress, oxidant stress, wounding, light stress, chemical stress and other pathogenic stresses.

In addition to the above it was also uncovered while reducing the present invention to practice that homoserine and/or its derivatives, when externally applied to plant tissues, induce the accumulation of phenols, which as described above, greatly contributes to the ability of a plant to withstand stress conditions and pathogen attack. Further detail of the above is given in Example 8 of the Examples section which follows.

Thus, it will be appreciated, that application of homoserine or its derivatives can be utilized to increase the stress tolerance of plants. Such an application can be effected by spraying the plant, preferably the canopy thereof, with a solution including homoserine or its derivatives and preferably a surfactant (e.g., Tween20 or the like). Alternatively homoserine or its derivatives can be provided within the irrigation water utilized for watering the plant. Alternatively, a fertilizer including a fertilizer base including, for

example, plant essential minerals and any other compounds typically included within commercial fertilizers utilized for growing plants, can also include homoserine or its derivatives in a concentration sufficient to increase plant tolerance to stress.

Thus according to another aspect of the present invention there is provided a method of increasing the stress tolerance of a plant, which method includes the application of homoserine or homoserine derivatives to plants or plant seedlings.

According to another aspect of the present invention there is provided a genetically modified plant expressing a recombinant acetylhomoserine sulfhydrylase (AHS). The nucleic acid sequence encoding the recombinant AHS can be of any origin e.g., from yeast fungi or bacteria, or may be a synthetic gene. For example, it can be encoded by the met25 gene of the yeast *Saccharomyces cerevisiae* [ENTREZ X04493, Sangsoda *et al.*, 1985; Kerjan *et al.*, 1986], met25 gene of *Schizosaccharomyces pombe* [Brzywczy and Paszewski, 1994], cDNA of the fungi *Acremonium chrysogenum* [ENTREZ E12441and U19394, Japanese Patent Application No. JP 1996336391-A1]; the metY of the bacteria *Leptospira meyeri* [ENTREZ Y10744, Belfaiza *et al.*, 1998] the cysD gene of the fungus *Emericella nidulans* [ENTREZ U19394] or the metB gene of the bacterium *Lactococcus lactis cremoris* [ENTREZ U79490, Duwat *et al.*, 1997]. As with the HAT transformed plant, unexpectedly, methionine content remained at a level similar to that of wild type plants.

In addition, transgenic plants co-expressing both the HAT and AHS coding sequences also contained wild type levels of methionine.

It will be appreciated that although the transgenic plants of the present invention failed to produce the expected results with respect to methionine production and/or accumulation, such plants can serve as a basis for further genetic manipulation in efforts to generate methionine rich plants.

All of the above genes have been cloned, sequenced and their protein products have been characterized. Based on sequence homology these genes can be used to isolate additional or alternative genes encoding for HAT or for AHS from other species using cloning method and strategies well known in the art. The sequences described in the above references and/or which are identified by their ENTREZ accession numbers are incorporated by reference as if fully set forth herein.

In order to increase the methionine content, several approaches can be under taken.

According to one approach, the genetically modified plant of the present invention expressing both recombinant HAT and AHS, can be transformed with a recombinant mutant AK which is less sensitive to feedback inhibition by lysine and/or threonine than the wild-type plant AK. The nucleic acid sequence encoding such recombinant AK may be of any origin, e.g. bacterial, or may be a synthetic gene. In a preferred embodiment, this mutant AK is of bacterial origin and is expressed by the *E. coli lysC* gene. This gene has been expressed, both constitutively and in a seed-specific manner, in transgenic tobacco plants [Shaul *et al.*, 1992; Karchi *et al.*, 1993; Galili, 1995; EP 0485970, which are incorporated by reference as if fully set forth herein].

According to another approach, the transgenic plants expressing the genes encoding HAT, AHS and preferably the recombinant mutant AK can also be utilized for further genetic manipulation utilizing gene knock-out or knock-in techniques. Standard methods known in the art can be used for implementing knock-in/knock-out procedure. Such methods are set forth in, for example, United States Patent Nos. 5,487,992, 5,464,764, 5,387,742, 5,360,735, 5,347,075, 5,298,422, 5,288,846, 5,221,778, 5,175,385, 5,175,384, 5,175,383, 4,736,866 as well as Burke and Olson, Methods in Enzymology, 194:251-270, 1991; Capecchi, Science 244:1288-1292, 1989; Davies *et al.*, Nucleic Acids Research, 20 (11) 2693-2698, 1992; Dickinson *et al.*, Human

Molecular Genetics, 2(8):1299-1302, 1993; Duff and Lincoln, "Insertion of a pathogenic mutation into a yeast artificial chromosome containing the human APP gene and expression in ES cells", Research Advances in Alzheimer's Disease and Related Disorders, 1995; Huxley *et al.*, Genomics, 9:742-750 1991; Jakobovits *et al.*, Nature, 362:255-261 1993; Lamb *et al.*, Nature Genetics, 5: 22-29, 1993; Pearson and Choi, Proc. Natl. Acad. Sci. USA, 1993, 90:10578-82; Rothstein, Methods in Enzymology, 194:281-301, 1991; Schedl *et al.*, Nature, 362: 258-261, 1993; Strauss *et al.*, Science, 259:1904-1907, 1993, WO 94/23049, WO93/14200, WO 94/06908 and WO 94/28123 also provide information.

Alternatively such plants can be further transformed with one or more additional methionine biosynthesis gene(s) in order to further increase methionine content. Such transformation can be employed by standard methodology which is further detailed hereinunder.

In yet another approach, the genetically modified plant of the present invention containing both recombinant HAT and AHS, and possibly a recombinant mutant AK which is less sensitive to feedback inhibition by lysine and/or threonine than the wild-type plant AK, can be transformed with a nucleic acid sequence encoding a recombinant methionine-rich protein. As used herein the phrase "methionine-rich protein" refers to a protein characterized in that at least 5 %, preferably up to 35% of its amino acids are methionine residues. An example of a methionine-rich protein is the 10 Kd zein protein from maize [described by Kirihara *et al.*, 1988; accession number AC X07535].

Numerous ways can be employed according to the present invention to provide a gene encoding a methionine-rich protein. One option is to use a cloned gene which is derived from a natural source and which encodes a methionine-rich protein. A number of methionine-rich plant seed storage proteins have been identified and their corresponding genes have been

isolated. Examples include, but are not limited to: (i) a corn-derived gene encoding a 15 kDa zein protein which contains about 15 % methionine by weight [Pederson *et al.*, 1986]; (ii) a corn-derived encoding a 10 kDa zein protein which contains about 30 % methionine by weight [Kirihara *et al.*, 1988]; (iii) a Brazil nut-derived gene encoding a seed 2S albumin which contains 24 % methionine by weight [Altenbach *et al.*, 1987]; (iv) a sunflower seed-derived gene encoding a methionine-rich 2S protein [Kortt *et al.*, 1991]; and (v) a rice-derived gene encoding a 10 kDa seed prolamin which contains about 25% methionine by weight [Masumura *et al.*, 1989].

Transgenic plants expressing such recombinant methionine-rich genes have already been described. For example, the high methionine 2S albumin from Brazil nut has been expressed in the seeds of transformed tobacco under the control of the regulatory sequences from a bean phaseolin protein gene. The protein was efficiently processed from a 17 kDa precursor to the 9 kDa and 3 kDa subunits characterizing the mature native protein. The accumulation of the methionine-rich protein in the tobacco seeds resulted in an up to 30 % increase in the level of methionine in the seeds [Altenbach *et al.*, 1989]. This methionine-rich storage protein has also been efficiently expressed in canola seeds [Altenbach *et al.*, 1992] and in lupins [Molvig *et al.*, 1997]. In another case, high levels of the seed-specific 15 kDa methionine-rich zein were obtained under the control of the regulatory sequences from a bean phaseolin storage protein gene in transformed tobacco plants. The signal sequence of the monocot precursor was correctly processed in these transformed plants [Hoffman *et al.*, 1987; Bagga *et al.*, 1995]. The sequences described in the above references or references therein are incorporated herein by reference.

The isolated polynucleotide can be introduced into plants using any conventional method of transformation or by viral infection. One such transformation method is the leaf-disk approach [Hosch *et al.*,1985] which, as

further exemplified in the Examples section that follows, has been successfully employed to implement other aspects of the present invention.

Thus, according to one preferred embodiment of the present invention there is provided a genetically modified plant co-expresses (i) a mutaaspartate kinase (AK) which is less sensitive to feedback inhibition by lysine and/or threonine than the corresponding wild-type plant; (ii) a recombinant homoserine acetyltransferase (HAT); (iii) a recombinant acetylhomoserine sulfhydrylase (AHS); and (iv) a recombinant methionine-rich protein. The recombinant AK, HAT and AHS and additional endogenous plant enzymes (see Figure 2) provide for an alternative pathway for the synthesis of methionine which is insensitive to end product inhibition or competition for carbon skeleton with the threonine pathway. The recombinant methionine-rich protein, in turn, provides for an in-plant trap for free methionine, assisting in preventing further metabolism of this amino acid and in maintenance of physiologically acceptable levels thereof as a free amino acid.

The genetically modified plants of the invention may be heterozygotes, hemozygotes or homozygotes.

The generation of homozygote transgenic plants co-expressing any subset or all of the above recombinant proteins is readily available by, for example, crossing heterozygotes or homozygotes (selfed) plants independently expressing these proteins. Thus, for example, a T0 heterozygote plant expressing HAT is selfed one or several times to generate progenies which are homozygotes. Similarly, a T0 heterozygote plant expressing AHS is selfed one or several times to generate progenies which are homozygotes. Likewise, a T0 heterozygote plant expressing aspartate kinase which is not subject to end product inhibition is selfed one or several times to generate progenies which are homozygotes. Similarly, a T0 heterozygote plant expressing a recombinant methionine-rich protein is selfed one or several times to generate progenies which are homozygotes. Homozygote progenies are then crossed to obtain

heterozygotes co-expressing any pair of proteins. These heterozygotes are then selfed to obtain homozygotes expressing that pair of proteins. Homozygotes expressing that pair of proteins are then crossed with homozygotes expressing one or more of the additional proteins, to thereby obtain heterozygotes which are selfed to obtain homozygotes co-expressing any subset or all of the above listed proteins. It will be appreciated that crossing of heterozygotes followed by appropriate selection of heterozygote progenies can be similarly implemented. Progenies identification and selection can, for example, be effected by PCR amplification of the introduced heterologous sequences (nucleic acid coding sequences, promoters), and/or by resistance to antibiotics, e.g., hygromycin.

An alternative approach consists in serially transgenizing a plant and its transgenic progenies by genes encoding the above proteins. Still alternatively, a nucleic acid construct harboring sequences encoding for all or any subset of the above recombinant proteins can be employed in a single transgenization process to provide a genetically modified plant expressing or co-expressing any subset or all of the above proteins.

It is expected that a genetically modified plant according to the present invention would contain at least 10 %, preferably at least 2 fold, more preferably at least 5 fold, more preferably at least 10 fold or more, total methionine as compared with a corresponding wild-type plant. Such a genetically modified plant further or alternatively contains at least 10 %, preferably at least 2 fold, more preferably at least 5 fold, more preferably at least 10 fold, more preferably at least 20-40 fold, most preferably at least 40-100 fold, optimally above 100 fold, more free arginine as compared with a corresponding wild-type plant.

The expression of each of the isolated polynucleotides employed according to the present invention in plants is carried out under the control of a suitable plant promoter. Promoters which are known or found to cause

transcription of selected gene or genes in plant cells can be used according to the invention. The particular promoter selected should be capable of causing sufficient expression to result in the production of an effective amount of a desired protein. Such promoters may be obtained from plants, plant pathogenic bacteria or plant viruses.

The promoter can be a constitutive promoter which is active in all or most plant tissues, a tissue-specific promoter which is active mostly in specific tissue or tissues, an inducible promoter which is induced under stress conditions, and a chimeric promoter. The phrase "tissue specific promoter" refers also to a developmental stage specific promoter.

There is a plurality of constitutive promoters known to express in plant tissues. Examples of constitutive promoters that can be used according to the invention include, but are not necessarily limited to, the 35S and 19S promoters of cauliflower mosaic virus (CaMV35S and CaMV19S) [Guilley *et al.*, 1982]; the full-length transcript promoter from the figwort mosaic virus (FMV34S) [U.S. Pat. No. 5,512,466] the promoter of cassava vein mosaic virus (CsVMV) [Verdaguer *et al.*, 1996]; the sugarcane bacilliform badnavirus promoter that is active both in monocots and in dicots [Tzafrir *et al.*, 1998]; promoters isolated from plant genes such as *Arabidopsis* ACT2/ACT8 actin promoter [An *et al.*, 1996]; *Arabidopsis* ubiquitin UBQ1 promoter, rice actin promoter [McElroy *et al.*, 1990] and barley leaf thionin BTH6 promoter [Holtorf *et al.*, 1995], and promoters obtained from T-DNA genes of *Agrobacterium tumefaciens* such as nopaline and mannopine synthases.

Particularly useful promoters for use in the present invention are tissue-specific promoters such as fruit or seed specific promoters. There are a plurality of tissue specific promoters known to express in plant tissues. Tissue specific promoters may be used according to the present invention to direct methionine overproduction in tissues consumed as food or feed, such as seeds in cereals and tubers in potatoes. Examples of seed-specific promoters include,

but are not limited to. the bean phaseolin storage protein promoter shown to be expressed in a seed-specific manner in transgenic tobacco plants [Sengupta-Gopalan, 1985]; DLEC and PHSβ promoters from Phaseolus [Bobb *et al.*, 1997]; zein storage protein promoter [Vicente-Carbajosa *et al.*, 1997]; conglutin gamma promoter from soybean [Ilgoutz *et al.*, 1997]; AT2S1 gene promoter [Roeckel *et al.*, 1997; ACT11 actin promoter from *Arabidopsis* [Huang *et al.*, 1997]; napA promoter from *Brassica napus* [Ellerstrom *et al.*, 1996]. Examples of other fruit or seed specific promoters include the E8, E4, E17 and J49 promoters from tomato [Lincoln and Fischer 1988], as well as the 2A11 promoter described in U.S. Pat. No. 4,943,674.

For the expression in potato tubers, a promoter derived from the potato patatin gene may be used.

Stress inducible promoters can also be employed in the present invention including, but not limited to, the light inducible promoter derived from the pea rbcS gene [Coruzzi *et al.*, 1984]; the promoter from the alfalfa rbcS gene [Khoudi *et al.*, 1997]; [McElroy *et al.*, 1990]; promoters active in drought, such as DRE promoter or MYC, MYB promoters [Liu *et al.*, 1998; Abe *et al.*, 1997]; a promoter active in high salinity, such INT, INPS or prxEa [Nelson *et al.*, 1998; Wanapu *et al.*, 1996]; a promoter active under osmotic shock, such as Ha hsp 17.7G4 or RD21 promoters [Coca *et al.*, 1996; Koizumi *et al.*, 1993]; a promoter active in dehydration such drought, salt and freezing such rd29A [Kasuga *et al.*, 1999]; a promoter active during mechanical wounding cause by insects or by fungi penetration to the tissue such pinII promoter [Deping *et al.* 1993]; a promoter activated by UV radiation and high level of ozone such as the stilbene promoter [Schubert, 1997], and a promoter active in cases of pathogenicity, such hsr303J or str246C [Pontier *et al.*, 1998; Perez *et al.*, 1997].

The constitutive, tissue-specific and inducible promoters used for expressing the recombinant proteins of this invention may be further modified,

if desired, to alter expression characteristics, thus generating chimeric promoters. For example, the CaMV35S promoter may be ligated to a portion of the ssRUBISCO gene which represses the expression of ssRUBISCO in the absence of light, to create a chimeric promoter which is active in leaves but not in roots. As used herein, the terms "CaMV35S", "FMV35S" or to this effect any other promoter include genetic variations of these promoters, e.g., chimeric promoters derived by means of ligation with operator regio, random or controlled mutage, addition or duplication of enhancer sequences, e.

In addition, at the 3' end of the promoter used in the invention, a short nucleic acid sequence for 5' mRNA non-translated sequence may be added which enhances translation of the mRNA transcribed from the chimeric gene. An example is the omega sequence derived from the coat protein gene of the tobacco mosaic virus [Galilee *et al.*, 1987].

Since the metabolic pathways responsible for the synthesis of both methionine and arginine are present mostly in the plastids, in one preferred embodiment of the present invention the recombinant proteins are preferably modified to include a transit peptide for plastid targeting. Nucleic acids encoding transit peptides are well known in the art since the genes encoding for all of the proteins which are destined to operate in the plant plastids include such sequences. A preferred transit peptide to translocate foreign expressed proteins into the chloroplasts is derived from the pea rbcS-3A gene [Fluhr *et al.*, 1986]. Plastid targeting is of particular importance for the mutant AK and the HAT proteins which are directly involved in the synthesis pathway(s). The protein AHS and the methionine-rich protein may be directed to any compartment, either the plastid or the cytoplasm. Alternatively, the polynucleotide constructs described hereinabove can be utilized to directly transform plastid DNA, see for example, Toriyama, K. *et al.* (1988) Bio/Technology 6:1072-1074.

The methodology exemplified herein below in the Examples section for tobacco can be readily used to genetically modify other crop plants. One ordinarily skilled in the art would know how to implement the methodologies described therein in plant species other than tobacco.

The genetically modified plant according to the present invention is preferably a crop. As used herein in the specification and in the claims section that follows, the term "crop" refers to any plant cultivar grown in a commercialized form. Such plants are typically grown in cultivated fields. Plants which form good candidates for the genetic manipulation according to the present invention are those known to be relatively poor in methionine. Such plants include, but are not limited to, mainly legumes, e.g. soybean, clover, alfalfa, pea, mung bean and *Vicia narbonensis;* cereals (both seeds and vegetative tissues), e.g. wheat, barley, rice, oats, maize, sorghum and forage grasses; *Solanaceae*, e.g. potato, tomato; canola and sunflower.

These plants are especially suitable for genetic manipulation as described herein because they are shown in the prior art to be amenable for transformation processes and/or viral infection.

In order to be introduced into and expressed in a plant, a gene must be engineered for plant transformation and expression. To introduce such a gene into a plant and to express it therein, a suitable chimeric gene and expression vector must be constructed. A typical chimeric gene for transformation into a plant will preferably include a promoter region, a heterologous nucleic acid coding sequence and a 3' non-translated polyadenylation site. A “heterologous coding sequence” means a structural coding sequence that is typically not native to the plant being transformed. with respect to the promoter means that the coding sequence does not exist in nature in the same gene with the promoter to which it is now attached. “Chimeric gene” means a novel non-naturally occurring gene which is comprised of parts of different genes. In preparing the expression vector (cassette), various nucleic acid fragments may

be manipulated as necessary to create the desired vector. This includes using linkers or adapters as necessary to form suitable restriction sites or to eliminate unwanted restriction sites or other like manipulations which are known to those of ordinary skill in the art. The promoters applicable for use with the present invention are described herein above. The 3' non-translated region contains a polyadenylation signal which functions in plants to cause the addition of polyadenylated nucleotides to the 3' end of a mRNA sequence. Examples of suitable 3' regions are the 3' transcribed, non-translated regions containing the polyadenylation signal of the tumor-inducing (Ti) plasmid genes of *Agrobacterium*, such as the nopaline synthase (NOS) gene, and plant genes like the 7s soybean storage protein genes and the pea E9 small subunit of the RuBP carboxylase gene (ssRUBISCO).

The RNA produced by the constructs of the present invention also preferably contains a 5' non-translated leader sequence. This sequence can be derived from the promoter selected to express the gene or genes, and can be specifically modified so as to increase translation of the mRNAs. The 5' non-translated regions can also be obtained from viral genes, from suitable eukaryotic genes, or from a synthetic gene sequence. The present invention is not limited to constructs wherein the non-translated region is derived from the 5' non-translated sequence that accompanies the promoter sequence of choice. Rather, the non-translated leader sequences can be part of the 5' end of the non-translated region of the native coding sequence for the heterologous coding sequence, or part of the promoter sequence, or can be derived from an unrelated promoter or coding sequence as discussed above.

In a preferred embodiment according to the present invention, the vector that is used to introduce heterologous nucleic acid sequences into the plant will comprise an appropriate selectable marker. In a more preferred embodiment according to the present invention the vector is a plant expression vector comprising both a selectable marker and an origin of replication. In

another most preferred embodiment according to the present invention the vector will be a shuttle vector, which can propagate both in *E. coli* (wherein the construct comprises an appropriate selectable marker and origin of replication) and be compatible for propagation or integration in the genome of the plant species of choice. In yet another embodiment, the construct comprises the promoter of choice, and the gene of interest is placed in a viral vector which is used to infect the cells. This virus may be integrated in the genome of the plant species of choice or may remain non-integrated.

While the nucleic acid sequences encoding the functional proteins is an essential element of the invention, it is modular and can be used in different contexts. The promoter of choice that is used in conjunction with this invention will comprise any suitable promoter as further detailed herein above. It will be appreciated by one skilled in the art, however, that it is necessary to make sure that the transcription start site(s) will be located upstream of open reading frame. In a preferred embodiment of the present invention, the promoter that is selected will comprise an element that is active in the particular host plant cells of interest.

These elements may be selected from transcriptional regulators that activate the transcription of genes essential for the survival of these cells in conditions of stress or starvation, including the heat shock proteins. Promoters containing this type of sequence may advantageously be used according to the present invention.

As further detailed herein above, nucleic acid sequences encoding the translational start site (ATG) of the gene to be expressed, will be placed downstream of the transcription start site(s). Any equivalent functional element selected from similar elements in one or another organism may be used as appropriate in the plant species of choice. Equivalent functional elements will include elements with synthetic bases, or elements found in other

genes of plants as well as elements found in genes of other unicellular or multicellular organisms.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above description, illustrate the invention in anon-limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures in recombinant DNA technology described below are those well known and commonly employed in the art. Standard techniques are used for cloning, DNA and RNA isolation, amplification and purification. Generally, enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like are performed according to the manufacturers' specifications. These techniques and various other techniques are generally performed according to Sambrook *et al.*, Molecular Cloning--A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989). Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

EXAMPLE 1

Nucleic acid constructs:

The coding DNA sequence of the yeast *S. cerevisiae* met2 (Figure 5b, SEQ ID NO:6) and met25 (Figure 5d, SEQ ID NO:8) provided in plasmids pM2-1 and pEMBLYr25, respectively, were kindly provided by Prof. Yolande Surdin-Kerjan (Gif-sur-Yvette, France). The expressed amino acid sequences of these genes are shown in Figures 5a (SEQ ID NO:5) and 5d (SEQ ID NO:7), respectively. Met2 was first described by Langin *et al.* (1986). Met2 and met25 were deposited in the ENTREZ database under accession numbers AJ001940, M15675 (met2) and X04493 (met25). The coding sequence of the gram negative bacteria *Leptospira meyeri* metX gene was kindly provided by Prof. Isable Saint Girons (Institute Pasteur, France) as an insert into the pb12 plasmid. The MetX coding sequence (ENTREZ accession number Y10744) which was first described by Bourhy et al. (1997), is shown in Figure 5f (SEQ ID NO:9), while the amino acid sequence thereof is shown in Figure 5e (SEQ ID NO:10).

The substrates and co-factors for HAT (encoded by the met2 gene of yeast or by metX of *L. meyeri*) and AHS (encoded by the met25 gene of yeast) enzymes are present in plant plastids.

Two nucleic acid constructs were constructed for the expression of the yeast met2 gene in plants. Both constructs contained a DNA segment encoding a plastid transit peptide for targeting the yeast HAT enzyme to plant plastids: one was designed to express the yeast HAT enzyme in the vegetative tissues of plants (Figure 4b, YEAST GENE – met2; PRO – CaMSV35SΩ) and the other in the plant seeds (Figure 4b, PRO – bean phaseolin storage protein promoter).

Four nucleic acid constructs were constructed for the expression of the yeast met25 gene in plants: two were similar to the above met2 constructs, except for the structural gene coding sequence (Figure 4b, YEAST GENE -

met25 coding for AHS), while the other two lacked the DNA segment encoding the plastid transit peptide. The latter two constructs (Figure 4a) are therefore designed to target the AHS enzyme to the cytoplasm. In the same Figure 4a can be made with the yeast met2 gene.

Two nucleic acid constructs were constructed for the expression of the bacterial metX gene in plants. The first construct is similar to above met2 construct, except that the structural gene coding sequence (Figure 4b) includes a DNA segment which targets the bacterial enzyme to plastids. The second nucleic acid construct lacked the DNA segment encoding the plastid transit peptide which targets the enzyme to the cytoplasm (Figure 4a).

1a. Preparation of nucleic acid constructs including the yeast met2 gene: A polymerase chain reaction (PCR) fragment corresponds to the met2 coding sequence was amplified from the pM2-1 plasmid using the following oligonucleotide primers: P1: 5'-GGCAATGCTGCATACTTTAA AATCGAAAACG-3' (SEQ ID NO:1), which introduced an *Sph*I restriction site containing the ATG translation-initiation codon of met2; and P2: 5'-TCAGCTTAAACTCCAATAGGAAGGC-3' (SEQ ID NO:2), which is complementary to the 3' end of the gene and contains the stop codon thereof. PCR conditions were 30 cycles of: 1 minute of denaturation at 95 °C; 1.5 minutes of annealing at 61°C; and 1.5 minutes of elongation driven by Expend DNA polymerase (Boehringer), performed at 72 °C. The resulting PCR product was ligated to a PCR-vector, pGMT (Promega). After digestion with *Sph*I and *Pst*I, the PCR fragment was subcloned to the CE and CEPH vectors [Shaul *et al.*, 1992; Karchi *et al.*, 1993] which were previously digested with the same enzymes.

The CE vector containing the coding sequence of *S. cerevisiae* met2 (herein "CEmet2") was designed according to Figure 4b to express the gene in vegetative tissues and to target the HAT protein to the plant plastids. It contains the constitutive promoter (PRO) CaMV35SΩ. A DNA sequence

coding for the transit peptide (TP) derived from the pea rbcS-3A gene was inserted between the Ω DNA and the met2 coding sequence. Downstream of the met2 sequence, a DNA fragment containing the first 700 bp of the 3' non-coding region of the *Agrobacterium tumefaciens* octopine synthase gene, harboring the transcription termination and polyadenylation signals of this gene (TER), was inserted. Pnos – promoter of nopaline synthase gene of *A. tumefaciens*; hpt – gene for hygromycin resistance; pAg7 – gene 7 poly(A) of *A. tumefaciens*.

The CEPH vector containing the coding sequence of *S. cerevisiae* met2 (herein "CEPHmet2") was designed according to Figure 4b to express the gene in seeds and to target the HAT protein to the plant plastids. It contains the bean phaseolin storage protein promoter (PRO). The other sequences are as for the CEmet2 vector above.

The DNA inserts were sequenced from both strands to ensure that no mutation had been introduced during the course of PCR amplification.

The CEmet2 and CEPHmet2 vectors were digested with the restriction endonucleases *Sma*I and *Sac*I and the inserts harboring the met2 gene and the other sequences described hereinabove were subcloned into the binary Ti plasmid, pGPTV-HPT-105, carrying the gene for hygromycin resistance [Becker *et al.*, 1992). As further detailed hereinunder, these constructs were used to transform tobacco plants by the leaf disc transformation method.

CDmet2 and CDPHmet2 vectors are designed according to Figure 4a without the DNA sequence coding for the transit peptide.

1b. Nucleic acid constructs including the yeast met25 gene: Four constructs including the yeast *S. cerevisiae* met25 gene were prepared. Two constructs according to Figure 4b for targeting of the resulting protein AHS to the plastids, herein designated CEmet25 and CEPHmet25, were similar to the CEmet2 and CEPHmet2 constructs described above, except for the structural gene which was met25 in this case, whereas the other two constructs were

designed according to Figure 4a for targeting of the resulting AHS to the cytosol, and were designated CDmet25 and CDPHmet25, respectively.

A polymerase chain reaction (PCR) fragment corresponding to the met25 coding sequence was amplified from the pEMBLYr25 plasmid using the following oligonucleotide primers: P3: 5'-GGCATGCCATCTCATTT CGATACTG-3' (SEQ ID NO:3), which introduced an *Sph*I restriction site containing the ATG translation-initiation codon of met25 and P4: 5'-TCATGGTTTTTGGCCAGCGAAAACAG-3' (SEQ ID NO:4), which is complementary to the 3' end of the gene and contains the stop codon thereof. PCR conditions were 30 cycles of: 1 minute of denaturation at 95 °C; 1.5 minutes of annealing at 61°C; and 1.5 minutes of elongation driven by the Expend DNA polymerase (Boehringer) at 72 °C. The resulting PCR product was ligated to the PCR-vector, pGMT (Promega). After digestion with *Sph*I and *Pst*I, the PCR fragment was subcloned to the CE and CEPH vectors as described above and to the CD and CDPH vectors.

1c. Preparation of nucleic acid constructs including the bacterial metX gene: Two constructs each including the bacterial *L. meyeri* metX gene were prepared. One construct was designed for targeting the resulting protein AHT to the plastids, herein designated CEmetX, was similar to the CEmet2 construct described above, except for the structural gene which was metX in this case, whereas the other construct was designed similarly to that shown in Figure 4a and results in targeting AHS to the cytosol (designated CDmetX).

A polymerase chain reaction (PCR) fragment corresponding to the metX coding sequence was amplified from the pb12 plasmid using the following oligonucleotide primers: P1: 5'-AGCATGCTTACCTCCGAACA GAACG-3' (SEQ ID NO:11), which introduced an *Sph*I restriction site containing the ATG translation-initiation codon of metX; and P2: 5'-

CGTAG -3' (SEQ ID NO:12), which is complementary to the 3' end of the

gene, and which includes the 6 x Histidine epitope tag (in frame), a stop codon and a *PstI* restriction site. PCR conditions were 30 cycles of: 1 minute of denaturation at 95 °C; 1.5 minutes of annealing at 58°C; and 1.5 minutes of elongation effected by the Expend DNA polymerase (Boehringer) and performed at 72 °C. The resulting PCR product was ligated to a PCR-vector, pGMT (Promega). After digestion with *Sph*I and *Pst*I, the PCR fragment was subcloned into the CE and CD vectors. The CDmetX vector is designed according to Figure 4a without the DNA sequence coding for the transit peptide.

1d. Yeast strains, media, growth condition and complementation assays: The following *Saccharomyces cerevisiae* strains were used while reducing the present invention to practice: CC360-16D (MATa, his7, ura3, met2) and CC365-1A (MATa, his3, ura3, met25) [described in Surdin-Kerjan *et al.*, 1989; Forlan *et al.*, 1991] were kindly provided by Prof. Yolande Surdin-Kerjan (Gif-sur-Yvette, France); the strain X4004-3A (MATa, lys5, trp1, ura3, met2) was kindly provided by Prof. Martegani (Milan, Italy).

To test whether the cloned PCR fragments of the met2 and met25 genes coded for active HAT and AHS enzymes, these PCR fragments were subcloned in a yeast expression vector which was then used in a complementation assay to transform yeast mutants not expressing either HAT or AHS, respectively.

Thus, the respective pGMT plasmids were digested by the restriction endonucleases *Nco*I and *Sal*I and the sticky ends of the inserts thereof were blunted by a Klenow enzyme (Boehringer). Each of the inserts was then independently subcloned into the yeast expression vector pFL61 [Minet *et al.* 1992], which was previously digested with the restriction endonuclease *Not*I and was blunted by Klenow. Met2 and met25 sense and antisense vectors were identified and isolated.

The sense and antisense orientation of the two yeast genes met2 and met25 were then transformed in yeast strains CC360-16D, X4004-3A and CC365-1A, as appropriate, and the transformed mutants were grown at 30 °C, under agitation, in 0.67 % Yeast Nitrogen Base (YNB, Difco) supplemented with 2 % glucose and the appropriate amino acids, without methionine. Yeast transformation was performed following a standard protocol [Vanoni. *et al.*, 1983]. All of the sense constructs complemented their respective mutant yeast strains, whereas all of the antisense constructs failed to complement these mutant yeast strains, indicating that the cloned PCR fragments of the met2 and met25 genes encode for active HAT and AHS enzymes, respectively.

1e. Nucleic acid constructs including the E. coli lysC gene: The *E. coli lysc* gene used herein expresses the mutant Ak-III isoenzyme. The gene has been cloned and sequenced [Cassan *et al.,* 1986]. The preparation of the DNA constructs comprising the *E. coli lysc* gene was carried out as described in EP 0 485 970, herein incorporated by reference.

Four nucleic acid constructs were constructed for the expression of the *E. coli lysC* gene. Two constructs contained the DNA sequence coding for the transit peptide (from pea rbcS-3A gene) for targeting of the expressed AK to the plastids (Figure 6), and two constructs lacked the DNA sequence coding for the transit peptide, thus targeting the expressed AK to the cytosol. In each of the cases, the promoter was CaMV35S or a tissue-specific promoter, for vegetative or tissue-specific expression of the mutant AK, respectively.

1f. Nucleic acid constructs including the maize gene coding for the 10 kDa zein: The coding sequence of the maize gene coding for the methionine-rich 10 kDa zein [described by Kirihara *et al.*, 1988; accession number AC X07535] was kindly provided by Dr. Suman Bagga (New Mexico State University, USA). Nucleic acid constructs including this gene are constructed under the constitutive promoter CaMV35S or under the inducible rbcS promoter of alfalfa, which expresses the protein in high level in vegetative

tissues (Figure 7). PCR is carried out with primers having the *SmaI* and *XbaI* restriction sites and the resulting PCR product is ligated to PCR vector pGMT (Promega). After the digestion with *XbaI* and *SmaI*, the PCR fragment is subcloned to the pPZP111 plasmid [Hajdukiewicz *et al.*,1994], containing the CaMV35S promoter or the rbcS promoter at the 5' end and the epitope tag 3HA at the 3' end, useful for further detection of the protein in the tissues by using suitable antibodies.

EXAMPLE 2

Plant transformation and analysis of transgenic plants:

Eight constructs including the yeast met2 and met25 coding sequences, namely, the constructs CEmet2, CEmet25, CEPHmet2, CEPHmet25, CDmet25 and CDPHmet25 or the bacterial metX constructs: CEmetX and CDmetX were used for transformation of *Nicotiana tabaccum* cv. *samson NN* using the leaf-disk protocol [Hosch *et al.*, 1985]. Transgenic plants were selected based on their ability to regenerate and root on media containing 15 mg/ml hygromycin. The plants were further grown on Nitsch medium in magenta boxes in a growth room at 25 °C, light intensity of 30 to 40 mE $\cdot$ m^{-2} $\cdot$ sec^{-1}, and 16/8 hours of day/night lengths. Leaves from about 1-month-old plants grown in magenta were tested for the presence of the yeast met2 and met25 genes or the bacterial metX gene by PCR using the primers and PCR conditions described herein above.

Thirty positively identified transgenic plants were then transferred to a greenhouse, grown to maturity, and selfed. The T1 progenies were tested for the segregation of the hygromycin-resistance gene that was included in the yeast or bacterial gene constructs. In all cases, approximately three-quarters of the T1 progenies were hygromycin-resistant, indicating that each of the transgenes, independently, was integrated at a single locus of the tobacco genome. Seeds from the T1 selfed transgenic plants were germinated and

grown on Nitsch medium containing hygromycin and were then grown to maturity in the greenhouse, after which seeds were collected therefrom.

EXAMPLE 3

Materials and methods

3a. Production of anti-HAT and anti-AHS antibodies: A 570 bp *Eco*RI-*Xho*I fragment from the yeast met2 gene and a 600 bp *Bam*HI-*Sal*I fragment from the yeast met25 gene were subcloned into plasmid pGEX-5T (Pharmacia) in-frame with the coding sequence of the glutathione S-transferase (GST) gene thereof. The fusion proteins were purified and were independently injected into rabbits following the instructions of the plasmid provider. The polyclonal antibodies against the fusion proteins were purified on affinity column (BioRad, Richmond, CA). Aliquots of eluted antibodies were stored at 4 °C in the presence of 0.05 % sodium azide.

3b. Western and protein dot blot analyses: Leaves of transgenic plants as well as of wild-type plants were homogenized with a mortar and pestle in equal volume of cold 100 mM Tris-HCl, pH 7.5, containing 2 mM EDTA and 1 mM phenylmethyl- sulphonylfluoride (PMSF). Following 5 minutes of centrifugation at 16,000 x g, at 4 °C, the supernatant was collected. Protein concentration was measured by standard procedure according to the Bradford method. Proteins were size-separated by SDS-PAGE by standard procedure according to Laemmli and Western or protein dot blots were then prepared. Western blots were prepared by standard procedure following the Burnette protocol. The resulting blots were reacted with the above described antibodies.

3c. Northern blot analysis: Total RNA was isolated from 250 mg samples of leaves of 2-month-old plants using Tri-Reagents (Sigma). RNA was electrophoresed and transferred to a nitrocellulose membrane (Hybond N, Amersham). The RNA blot was pre-hybridized and washed according to the protocol provided by the membrane manufacturer. Denatured, full-length, ^{32}P-

dCTP labeled (Redi-prime kit, Amersham) yeast met2, met25, or bacterial metX probes were used for hybridization.

3d. Measurements of free amino acid levels in leaves and seeds of transgenic plants: Axenic tobacco plants grown for about 1 month on Nitsch medium in magenta boxes were harvested, their leaves were ground in liquid nitrogen and kept frozen. Free amino acids were extracted from a sample of frozen leaves or from seeds by standard procedures. Tissue (approximately 200 mg) was homogenized in a mortar and pestle in the presence of 600 ml of water:chloroform:metanol (3:5:12, v/v). Following short centrifugation, the supernatant was collected and the residue was extracted with 600 ml of the same extraction reagent. Following centrifugation, the two supernatants were combined. Chloroform (300 ml) and water (450 ml) were added to the supernatant, the resulting mixture was centrifuged, and the upper water-methanol phase was collected, dried, and dissolved in 200 ml of H_2O. The concentration of free amino acids was determined using the O-phthalaldehyde reagent, followed by measuring the 335/447 nm fluorescence. Amino acid composition was determined by loading a sample of 66 nmol of total free amino acids on a Hewlett-Packard Amino Quant Liquid Chromatograph.

3e. Measurements of total phenols levels in leaves of transgenic plants: Homozygous plants grown for 10 weeks in greenhouse were harvested. One gram of leaf No. 5 (numbered from the shoot tip) was extracted with 50% ethanol for phenol detection. The extract was heated for 15 minutes at 80°C and then centrifuged at 4,000 *g* for 10 min. Ethanol (50%) was added to the supernatant to final volume of 10 ml, from which 1 ml was collected and mixed with 0.8 ml folin reagent and Na_2CO_3. The reaction produced a blue color, detectable by spectrophotometer at 725nm; phenol quercitin was used as a standard.

3f. Synthesis of O-acetylhomoserine: O-acetylhomoserine was synthesized following the protocol of Nagai *et al.* [1971]. Its biological

activity was tested by the ability of the yeast strain CC360-16D (met2-) to grow in minimal medium supplemented with the synthesized O-acetylhomoserine.

3g. Analysis of transgenic plants: T0 transgenic plants expressing the yeast met2 or met25 gene, either constitutively or in a seed-specific manner, and the transgenic plants expressing the metX gene were analyzed by PCR for the presence of the yeast sequences using met2, met25 or metX specific primers. All thirty transgenic plants for each of the chimeric constructs which were selected for hygromycin resistance were found to carry the respective yeast HAT or AHS gene. Six plants of each group that expressed the highest level of their respective gene or enzyme were self-pollinated (selfed), and T1 plants were divided to heterozygous and homozygous plants, on the basis of germination of their selfed T2 seeds on medium with hygromycin. While all of the T2 control plants were sensitive to hygromycin, all of the T2 seeds of the homozygous progenies were hygromycin-resistant. Most of the T2 seeds of heterozygous plants segregated as 0.75 resistant and 0.25 sensitive to hygromycin. The segregation frequency of the T1 plants also indicated that the T-DNA behaved as a single Mendelian locus in these plants.

Western and protein dot blot analyses were used to detect the two yeast proteins with the polyclonal antibodies as described above.

3h. Testing transgenic plants expressing the met2 gene for paraquat resistance: Leaf No. 5 was removed from homozygous transgenic plants and from untransformed plants grown for ten weeks in the greenhouse. Fifteen disks (2 cm in diameter) were excized from each leaf. Five disks were transferred to plates containing DDW with 0.05% triton X, and ten disks were transferred to two plates containing 10^{-5} mM paraquat (Zeneca, Fernhrst, Surrey, UK) with triton X (five disks per plate). Following one hour in the dark, the plates were exposed to sunlight for 4 hours. The disks were wiped dry and placed in tubes containing 80% acetone. After grinding the leaves in a

homogenizer, the tubes were shaken overnight at 4°C. The chlorophyll level was then measured by spectrophotometry and calculated according to the following:

$$\mu g \text{ chlorophyll/g tissue} = [O.D._{663} \times 8.02 + O.D._{645} \times 20.02] \times V \times 10^{-3}$$

3i. Testing transgenic plants expressing the met2 gene for tobacco mosaic virus (TMV) resistance: Transgenic homozygous plants and untransformed plants were grown in a greenhouse for 6 weeks, to a five leaf stage. The shoot tips were cut, and the leaves removed, except for leaf No. 3 (10 cm in length). The plants were infected with a suspension of the common strain of TMV (0.5 mg/ml) in a solution containing carborundum, by gently rubbing 50 µl of the virus suspension on to the adaxial surface of the leaf. Control plants were treated in identical fashion with DDW and carborundum. After four days of growing in the green house, the number and the size of the lesions were measured.

3j. Testing transgenic plants expressing the met2 gene for an increase in total phenols as a result of exposure to UV-B light: In order to test the ability of the transgenic plants (line 17) and untransformed plants to accumulate phenols during exposure to UV-B light, ten-week plants were transferred from the greenhouse to a dark room. A UV-B fluorescent light source (302 nm) was placed 1.5 m from the plants. Samples from leaves 4 and 5 were taken prior to exposure and again after exposures of 3, 6, and 12 h. Total phenols and antioxidant activity were measured.

3k. Testing transgenic plants expressing the met2 gene for tolerance to drought stress: Transgenic line 17 and untransformed plants were grown for eight weeks in the greenhouse. Irrigation of half the plants was stopped in order to generate drought stress in these plants. Leaves and plants were removed on the first the day of the experiment, following eight days, and at morning wilting (i.e. when the upper leaves were already hanging loosely in the morning). The level of proline (amino acids that accumulate during

osmotic stress) was measured in these leaves according to protocol [Bates *et al.*, 1973]. Osmotic adjustment was examined by placing leaves in DDW for 4 h in a cold room, freezing them in liquid nitrogen and measuring total solubles by psycrometer. A low level of osmotic potential indicates a high level of osmolites in the cell.

EXAMPLE 4

Transgenic plants expressing the yeast met2 gene

The expression of the met2 gene in vegetative tissues of T3 homozygous plants was tested by Northern blot analysis, using the yeast met2 gene as a probe. Figure 8a shows the results of several representative transgenic plants showing high and very low expressions. No band appears in untransformed plants tested, while a band corresponding in size to the met2 mRNA (1650 bases) appears at different intensities in the various transgenic plants tested. Western dot-blot analysis, using antibodies raised against yeast HAT, was performed in order to examine the level of the HAT protein in leaves (Figure 9a). The HAT protein was present in the transgenic plants and, as expected, there was a positive correlation between the protein and mRNA levels (compare Figures 8a and 9a).

The transgenic plants expressing the met2 gene under the control of the CaMV35SΩ promoter apparently had a normal phenotype. In order to examine the product of the HAT enzyme, i.e., *O*-acetylhomoserine, the level of free amino acid was determined in extracts of leaves of the transgenic plants. Unexpectedly, it was found that these plants overproduced arginine and ornithine: in some plants the level of arginine reached 64 mol % of the total free amino acids (see Table 1, below). However, no *O*-acetylhomoserine could be detected. It is possible that this metabolite is further catabolized in the plant cells. Transgenic plants homozygous for the met2 transgene had about twice the level of arginine of those heterozygous for the transgene

(Table 1. below). suggesting that arginine overproduction correlates with the expression level of the met2 transgene. Arginine accumulation in the homozygous progeny of transgenic plant No. 11 was elevated about 30-fold than in control, untransformed plants (Table 1). Total phenols accumulation in the transgenic plants expressing the met2 gene was approximately 1.5-fold higher than that of W.T. plants (Figure 12a).

TABLE 1

Percent arginine and ornithine from the total free amino acids in transgenic plants expressing the yeast met2 gene.

Plant	Arginine	Ornithine
Wild-type	1.9	0
T17 (heterozygous)	15.3	0.6
T17 (homozygous)	38.7	2.2
T12 (heterozygous)	11.4	0.6
T12 (homozygous)	27.7	9.4
T18 (heterozygous)	10.7	1.2
T11 (homozygous)	64.1	1.7

T - Transgenic

Tobacco plants were also transformed with a construct harboring the yeast met2 coding sequence under the regulation of the seed-specific promoter of bean phaseolin, that is active both in the embryo and, to a smaller extent, in the endosperm of tobacco seeds. The expression of the met2 gene was assayed in protein dot blots derived from mature seeds of 30 individually transformed heterozygous T0 plants, as well as from three control untransformed plants. Protein extracts from several of the transgenic seeds showed a positive spot reactive with anti-HAT serum. The level of met2 protein in mature seeds was generally lower than in the vegetative tissues (compare Figures 9a and 9b). Free amino acids were examined from four of the transgenic seeds and one of these plants, No. 325, contained 51 mol % arginine and 0.5 mol % ornithine.

EXAMPLE 5

Transgenic plants expressing the yeast met25 gene

The four constructs including the met 25 gene, namely, CEmet25, CEPHmet25, CDmet25 and CDPHmet25, were subcloned into the binary vector pGPTV-HPT-105 of *Agrobacterium tumefaciens* and were introduced into *Nicotiana tabaccum* by the leaf-disc protocol.

To test for the expression of AHS under control of the CaMV35SΩ promoter, leaves from transgenic plants, grown for about 2 months in the greenhouse, were harvested, and protein extracts were reacted with anti-yeast AHS antibodies in Western blots. Out of 30 transgenic plants, 25 showed a positive band with the expected size of AHS (52 kDa). Representative transgenic plants expressing the yeast AHS are shown in Figure 10a. The co-migration of the AHS polypeptide in both the chloroplastic and cytoplasmic types of transgenic plants indicates that in the chloroplastic-type the transit peptide had been removed and the enzyme was apparently translocated into the plastids. Generally, plants in which AHS was transported to the plastids showed higher AHS levels than plants expressing this enzyme in the cytosol.

Seeds from transgenic plants expressing the yeast AHS protein under the control of the seed-specific phaseolin promoter also exhibited cross-reacting bands with the expected size of the yeast protein (Figure 10b). However, no significant differences in protein levels were evident between the proteins present in the cytosol and in the plastids (Figure 10b). Transgenic plants expressing the yeast met25 gene had a normal phenotype and their free amino acid composition was identical to that of control, untransformed plants.

EXAMPLE 6

Transgenic plants co-expressing both the met2 and the met25 yeast genes

Tobacco plants producing both yeast AHS and yeast HAT were obtained by crossing transgenic plants producing each of these enzymes

individually. Seeds from crossed pods were germinated and grown on media containing hygromycin. Approximately 25 % of the seedlings derived from each cross exhibited PCR positive results for both genes, and were referred to as the progenies of the cross. Three transgenic genotypes were used: plant 17, which produced a high level of the yeast HAT in the plastids; plant 110, which produced a high level of AHS in the cytosol, and plant 205, which produced a high level of AHS in the plastids. Two crosses were performed: 17 x 110 and 17 x 205. Eight plants were examined by protein dot-blot analysis with antibodies against yeast AHS (Figure 11a) and against yeast HAT (Figure 11b), showing that the progenies co-produced both AHS and HAT.

EXAMPLE 7

7a. Transgenic plants expressing the bacterial metX gene: The expression of the met2 gene in vegetative tissues of T0 heterozygous plants was tested by Northern blot analysis by using the yeast metX gene as a probe. Figure 8b illustrates the results of several representative transgenic plants. Reactive bands were not detected in the untransformed plants tested while a band corresponding in size to the metX mRNA was detected at different intensities in the various transgenic plants. These plants accumulate arginine up to 15% of their total free amino acids and displayed phenol levels approximately 1.5-fold higher than that of W.T. plants (Figure 12b).

7b. Transgenic plants expressing the yeast met2 gene - resistance to paraquat: Disks leaves from representative homozygous transgenic plants expressing the met2 gene and from untransformed plants were exposed to paraquat. The results demonstrate a correlation between the level of met2 expression in transgenic plants and the effect of paraquat. Transgenic lines 11 and 12 that express the met2 at high level are more resistant to paraquat than the untransformed plants (Figure 13). The leaf disks acquired from

transformed lines 11 and 12 remained green while those acquired from the untransformed plants whitened as the results of paraquat effect.

7c. Transgenic plants expressing the yeast met2 gene - resistance to tobacco mosaic virus (TMV): The results show (Figures 14a-b) that the number and diameter (1.5-2.5 mm) of the TMV lesions in transgenic line 12 which expresses the met2 at high levels is significantly lower than in untransformed plants (wild-type). In transgenic line 17 which expresses the met2 gene at moderate levels, lesion density and diameter (2.5-3.5 mm) was less than that observed in untransformed plants, but higher than that observed in line 12.

7d. Transgenic plants expressing the yeast met2 gene accumulate phenols at high rate: Ultra-violet-B light (UV-B - 280-320 nm) damages living organisms. Plants protect themselves by producing flavonoid compounds, which accumulate in the epidermal cells and absorb the UV light. It was found that UV-B causes oxidative stress and that phenolic compounds can be activated by the absorption of UV-B light.

UV-B exposure on leaves generates an increase in total phenols. Transgenic line 17 accumulated twice as much phenol as the control, while the W.T. plants accumulated 1.3 times of the control (Figure 15).

7e. Transgenic plants expressing the yeast met2 gene- tolerance to drought stress: Transgenic line 17 and wild-type plants were grown for eight weeks in the greenhouse. Irrigation of half the plants was stopped in order to cause drought stress in these plants. Leaves and plants were removed on the first the day of the experiment (Figure 16a), after eight days (Figure 16b), and at morning wilting (i.e. when the upper leaves were already hanging loosely in the morning) (Figure 16c).

The wild-type plants exhibited morning wilting on day 12 while transgenic line 17 exhibited morning wilting on day 14. This two day difference is statistically significant since during these days the biomass

accumulation (calculated as % Dry weight /Fresh Weight) of the plants were 7.7 ± 0.8 for the wild-type and 9.8 ± 1.1 for line 17, showing that line 17 can accumulate more biomass during stress. During the drought experiment, 21 times the amount of the amino acid proline accumulated in line 17 as compared to irrigated plants, while in line 18 only 7 times the amount accumulated. The result shows that line 17 accumulated 20% more solutes than the wild-type plants during wilting (Figures 16a-c), in order to protect against drought, which possibly explains the ability of line 17 to wilt two days later.

7f. Transgenic plants expressing the yeast met2 gene- tolerance to salt stress: Transgenic line 12 and wild-type plants were grown for five weeks in vermiculite and then transferred to hydroponics containers containing a growth medium. Following a week of adjustment, salt was gradually added over two days, to final concentrations of 75 and 150 mM NaCl. The control consisted of plants grown without the addition of salt. After two weeks line 12 showed more tolerance to salinity stress than wild-type plants: their leaves were greener and they showed less decay. The results of this preliminary experiment are summarized in the Table 2 below.

TABLE 2

The effect of salt on wilting of transgenic lines 12 and 18 10 days following salt application. Numbers in the table indicate the % of death and are an average of eight plants.

150 mM NaCl	75 mM NaCl	Without salt	
50% (0% green)	10%	0%	Wild-type
25% (60% green)	0%	0%	Line12

7g. Transgenic plants expressing the yeast met2 gene- tolerance to cold stress: Five, 35 day old plants from each transgenic line tested and from untransformed plants were transferred to a cold room at -1°C. After ten days the plants were returned to the greenhouse for recovery. Table 3 below shows

the ability of these plants to survive. The results show that line 12 is more tolerant to cold than the wild-type plants.

TABLE 3

The survival of transgenic plants after ten days at -1 °C. The numbers are average of five plants.

Transgenic line	Wild-type	19	17	14	11	12
Survival	0 (0%)	0 (0%)	2 (40%)	1 (20%)	1 (20%)	5 (100%)

EXAMPLE 8

Homoserine derivatives increase the level of total phenols in untransformed plants

The effect of several homoserine derivatives on phenol levels in wild type plants was examined. Leaf No. 5 of untransformed plants was removed and placed in tubes containing various homoserine derivatives and other control amino acids at 1 or 10 mM concentrations. Total phenols were measured. The results show an increase in total phenols which is roughly proportional to the rise in concentration of the homoserine metabolites. These results are not seen when glutamine or aspargine are applied (Figure 17).

Future experiments, which will employ for example, homoserine derivative plant spraying, will be conducted in efforts to test the ability of these compounds to confer systemic acquired resistance and as such to confer pathogen resistance similar to beta butyric acid protection previously described [Cohen *et al.*, 1994]. This would offer novel approaches for protecting seedling and plants from, for example, wilting disease and other forms of biotic and abiotic stresses.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications

cited herein are incorporated by reference in their entirety. In addition, IL 130014 is incorporated herein by refernce in its entirety. Citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

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WHAT IS CLAIMED IS:

1. An isolated polynucleotide comprising:

(a) a first nucleic acid sequence encoding an enzyme having homoserine acetyltransferase (HAT) activity or a second nucleic acid sequence encoding an enzyme having acetylhomoserine sulfhydrylase (AHS) activity; and

(b) a third nucleic acid sequences capable of enabling the expression of HAT or AHS in plant cells and, optionally, the transportation of the expressed HAT or AHS to the plastids.

2. The isolated polynucleotide of claim 1, comprising both said first and said second nucleic acid sequences.

3. The isolated polynucleotide of claim 1, wherein said third nucleic acid sequence includes:

(i) a plant promoter; and

(ii) a plant polyadenylation and termination sequence.

4. The isolated polynucleotide of claim 1, wherein said third nucleic acid sequence includes:

(i) a plant promoter;

(ii) a plant polyadenylation and termination sequence; and

(iii) a sequence encoding a plastid transit peptide translationally fused to the 5'-end of said first or said second nucleic acid sequence for the transportation of the expressed HAT or AHS to the plastids.

5. The isolated polynucleotide of claim 1, wherein said plant promoter is selected from the group consisting of a constitutive promoter, a tissue specific promoter, an inducible promoter and a chimeric promoter.

6. The isolated polynucleotide of claim 5, wherein said constitutive plant promoter is selected from the group consisting of CaMV 35S promoter, CaMV19S promoter, FMV34S promoter, sugarcane bacilliform badnavirus promoter, CsVMV promoter, *Arabidopsis* ACT2/ACT8 actin promoter, *Arabidopsis* ubiquitin UBQ1 promoter, barley leaf thionin BTH6 promoter, and rice actin promoter.

7. The isolated polynucleotide of claim 5, wherein said tissue specific plant promoter is selected from the group consisting of bean phaseolin storage protein promoter, DLEC promoter, PHSβ promoter, zein storage protein promoter, conglutin gamma promoter from soybean, AT2S1 gene promoter, ACT11 actin promoter from *Arabidopsis,* napA promoter from *Brassica napus* and potato patatin gene promoter.

8. The isolated polynucleotide of claim 5, wherein said inducible promoter is selected from the group consisting of a promoter induced in stress conditions comprising light, temperature, drought, high salinity, osmotic shock, oxidant, chemical or pathogenic stress, being selected from the light-inducible promoter derived from the pea rbcS gene, the promoter from the alfalfa rbcS gene, the promoters DRE, MYC and MYB active in drought; the promoters INT, INPS, prxEa, Ha hsp17.7G4 and RD21 active in high salinity and osmotic stress, the promoters hsr303J and str246C active in pathogenic stress; the rd29A promoter active in dehydration induced by drought, salt loading and by freezing; the stilbene promoter active during UV radiation and

high level of ozone, and the promoters hsr303J and str246C active in pathogenic stress.

9. The isolated polynucleotide of claim 1, wherein said first or said second nucleic acid sequence is derived from yeast or from bacteria, or is a synthetic gene.

10. The isolated polynucleotide of claim 1, wherein said first nucleic acid sequence includes a sequence as set forth in SEQ ID NO:5 or a functional portion thereof.

11. The isolated polynucleotide of claim 1, wherein said second nucleic acid sequence includes a sequence as set forth in SEQ ID NO:6 or a functional portion thereof.

12. An expression vector comprising the isolated polynucleotide of claim 1.

13. A transgenic plant, plant derived tissue or a plant cell comprising the isolated polynucleotide of claim 1.

14. A genetically modified plant comprising the isolated polynucleotide of claim 1, having an improved tolerance to a stress condition as compared with a corresponding wild-type plant, said stress condition is selected from the group consisting of high salinity stress, drought stress, temperature stress, mineral deficiency stress, osmotic stress, oxidant stress, chemical stress and pathogenic stress.

15. A genetically modified plant comprising the met2 gene of *Saccharomyces cerevisiae* or the metX gene of *Leptospira meyer*.

16. A genetically modified plant having an increased free content, as compared with a corresponding wild-type plant, of arginine and ornithine and/or its related metabolites selected from the group consisting of citrulline, putrescine, nitric oxide, spermine and/or spermidine.

17. A genetically modified plant having an increased free content, as compared with a corresponding wild-type plant, of phenols and/or phenols related metabolites.

18. A method for producing a genetically modified plant having an increased free content, as compared with a corresponding wild-type plant, of arginine and ornithine and/or related metabolites, the method comprising the steps of:

(a) transforming plant cells with an isolated polynucleotide capable of expressing in plants, an enzyme having homoserine acetyltransferase (HAT) activity or an enzyme having acetylhomoserine sulfhydrylase activity or both; and

(b) regenerating transformed plants from the transformed plant cells of (a) and selecting for plants that express HAT.

19. A method for producing a genetically modified plant having an improved tolerance to a stress condition as compared with a corresponding wild-type plant, the method comprising the steps of:

(a) transforming plant cells with an isolated polynucleotide capable of expressing in plants, an enzyme having homoserine acetyltransferase (HAT) activity; and

(b) regenerating transformed plants from the transformed plant cells of (a) and selecting for plants having an improved tolerance to a stress condition as compared with a corresponding wild-type plant.

20. The method of claim 19, wherein said enzyme having homoserine acetyltransferase (HAT) activity is selected from the group consisting of yeast met2 and bacterial metX.

21. The method of claim 19, wherein said stress condition is selected from the group consisting of high salinity stress, drought stress, temperature stress, mineral deficiency stress, osmotic stress, oxidant stress, chemical stress and pathogenic stress.

22. The method of claim 19, wherein said step of selecting (step c) is effected by growing said transformed plants under said stress conditions.

23. A method of increasing the stress tolerance of a plant, the method comprising the step of applying to the plant homoserine or homoserine derivatives.

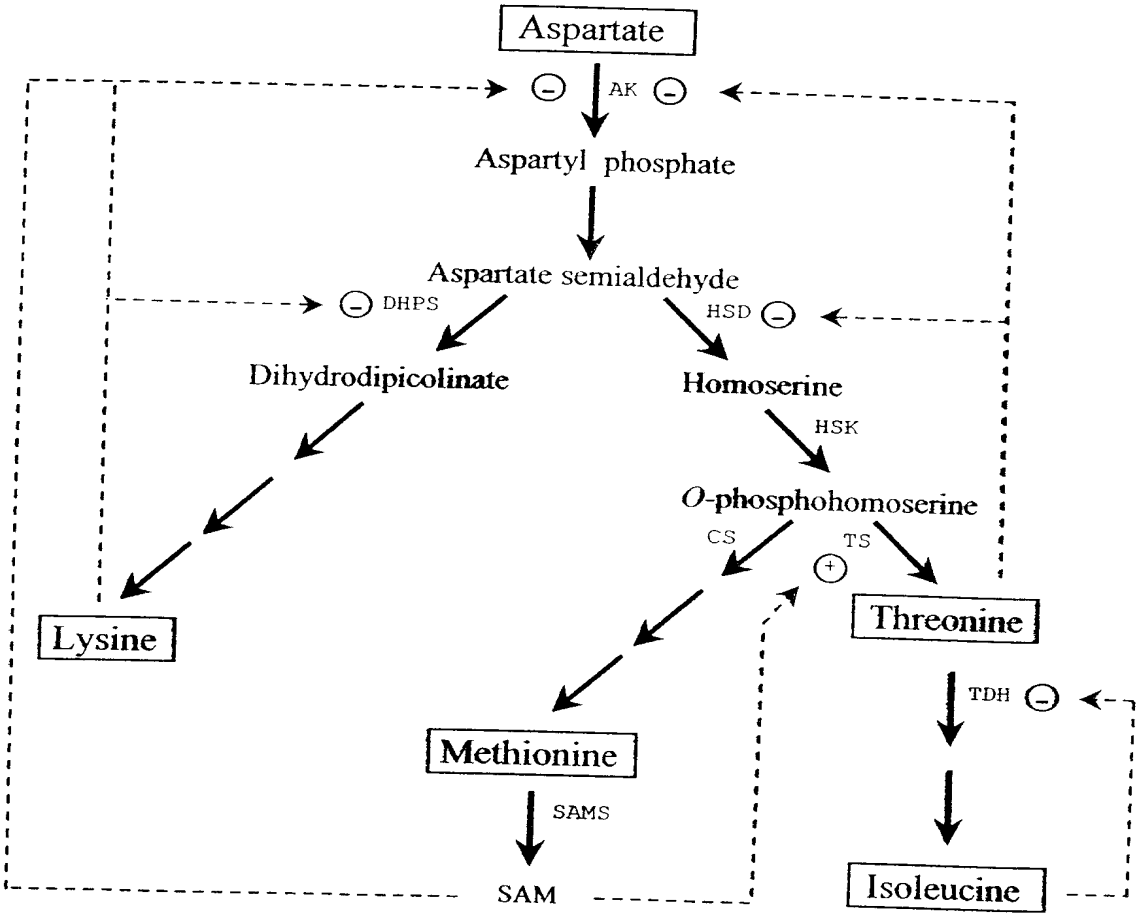
24. The method of claim 23, wherein said homoserine or homoserine derivatives are externally applied to the plant.

25. The method of claim 23, wherein said homoserine or said homoserine derivatives are provided in a liquid solution.

26. The method of claim 25, wherein said liquid solution also includes a surfactant.

27. A plant fertilizer comprising a fertilizer base and homoserine or homoserine derivatives, wherein said homoserine or said homoserine derivatives are provided in a concentration sufficient to induce stress tolerance within a plant growing on the plant fertilizer.

Fig. 1


Aspartate
AK
Aspartyl phosphate
Aspartate semialdehyde
DHPS
HSD
Dihydrodipicolinate
Homoserine
HSK
O-phosphohomoserine
CS
TS
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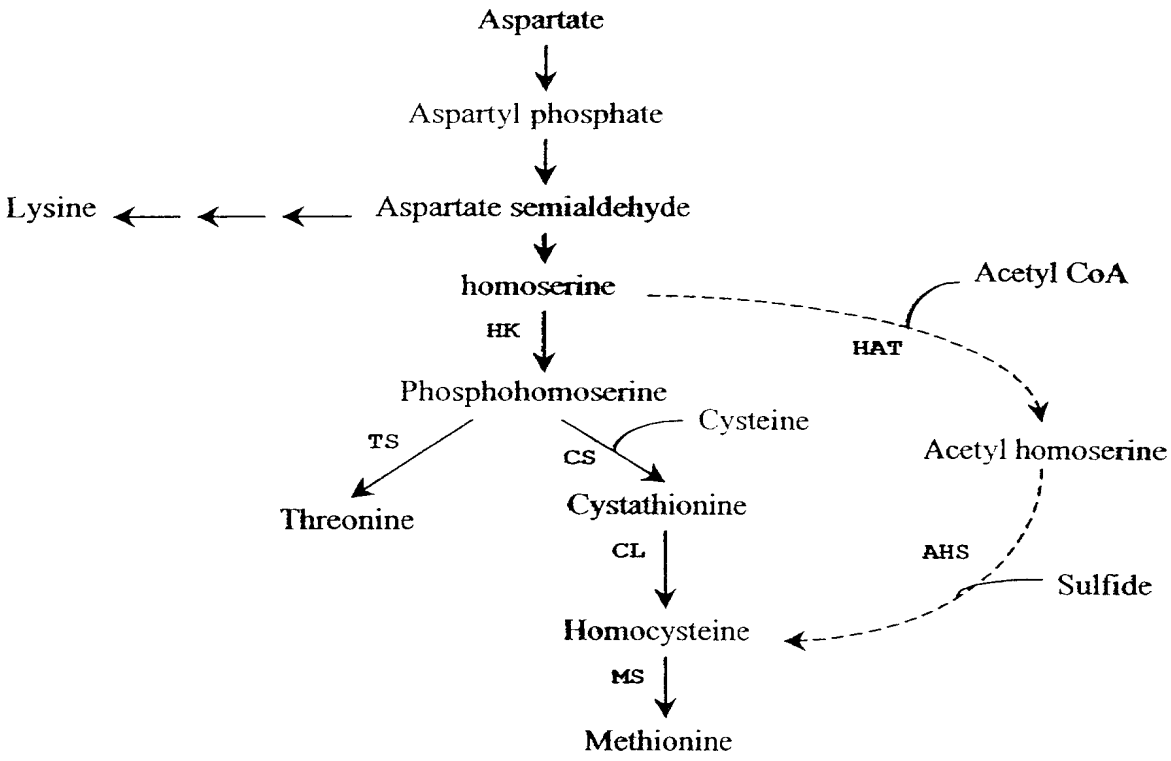
Threonine
TDH
Methionine
SAMS
SAM
Isoleucine

Fig. 2a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Aspartyl phosphate
Lysine
Aspartate semialdehyde
homoserine
Acetyl CoA
HK
HAT
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Cystathionine
CL
AHS
Sulfide
Homocysteine
MS
Methionine

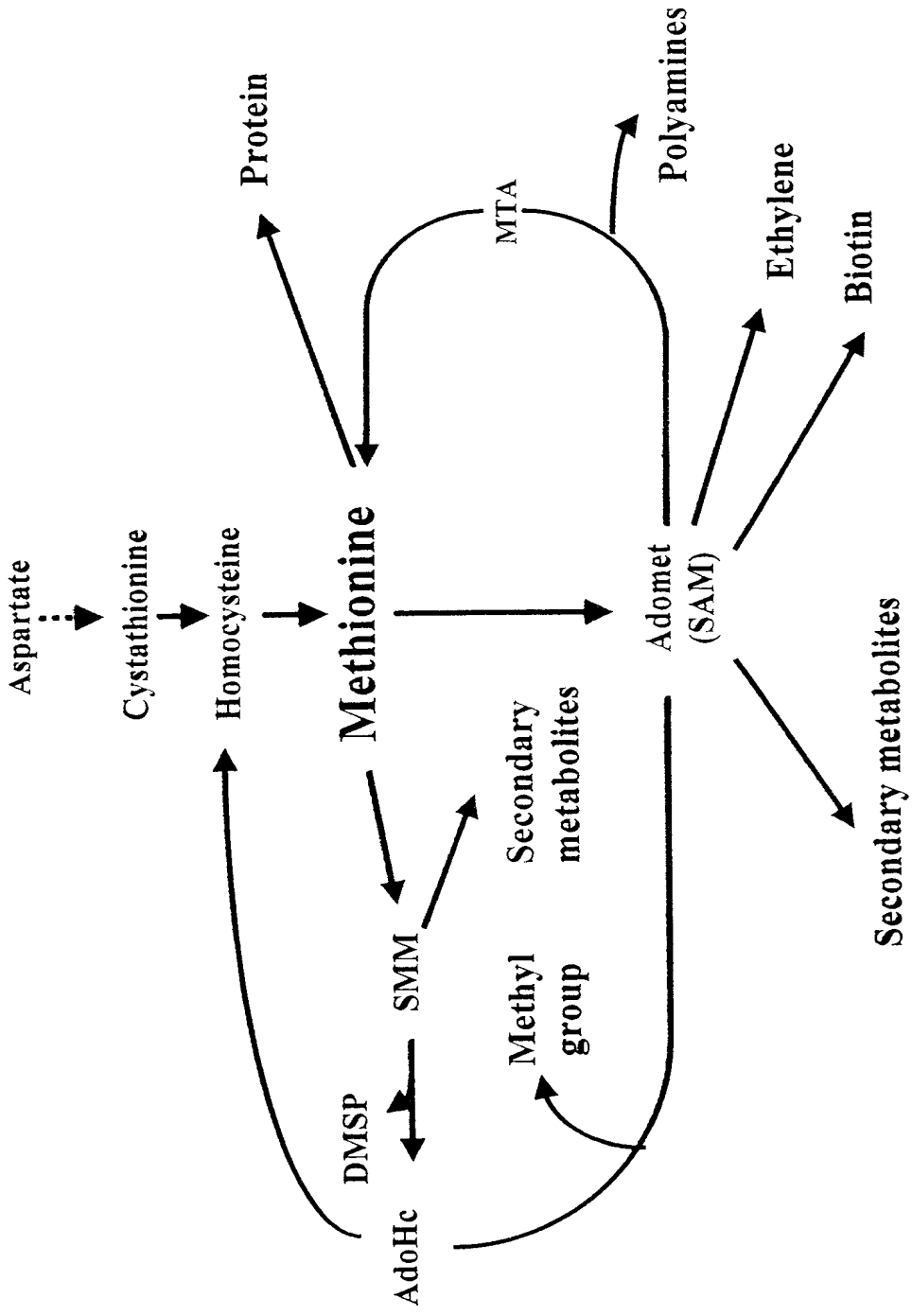
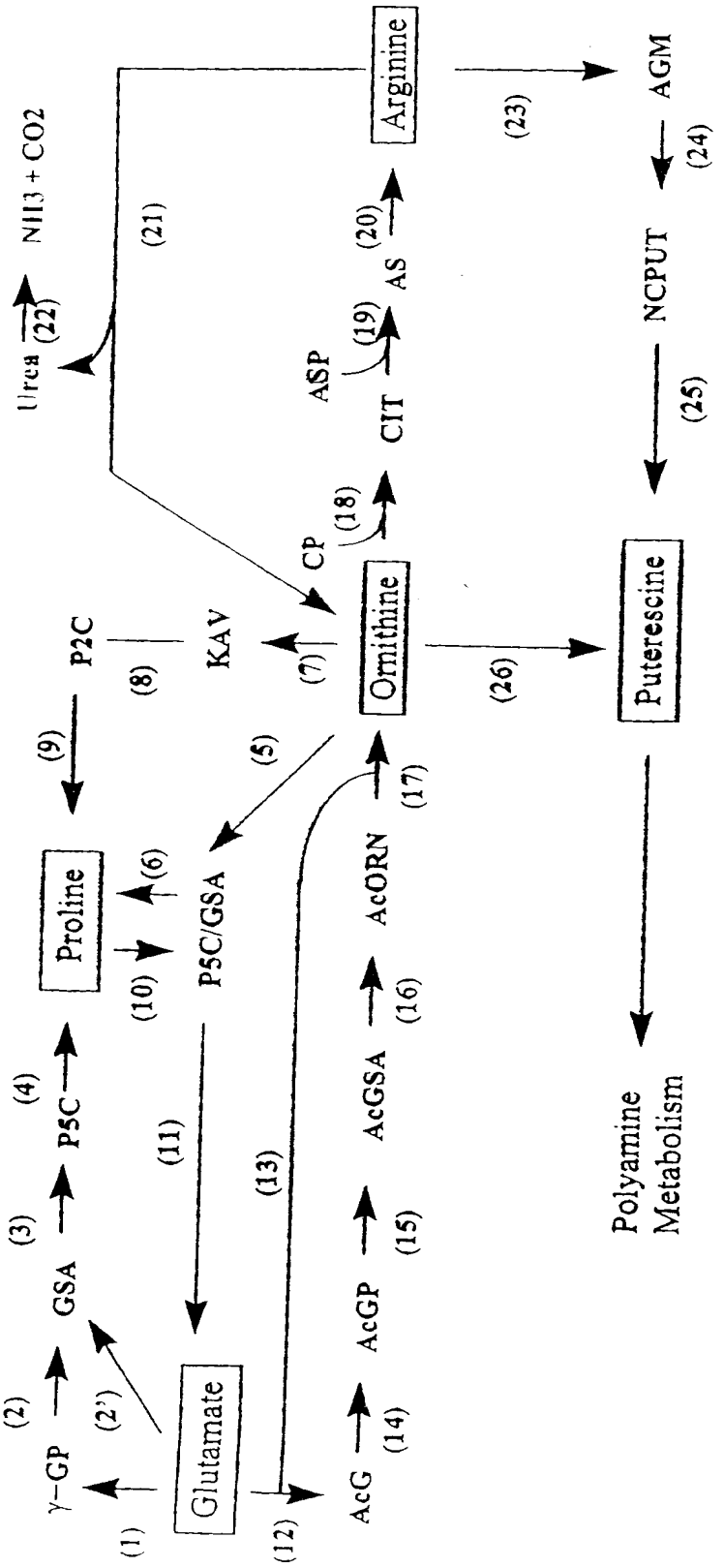

Aspartate
Cystathionine
Homocysteine
Methionine
Protein
MTA
Polyamines
Ethylene
Biotin
Adomet (SAM)
Secondary metabolites
Secondary metabolites
SMM
DMSP
AdoHc
Methyl group

Fig. 2b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γ-GP
GSA
P5C
Proline
P5C/GSA
P2C
KAV
Ornithine
CP
CIT
ASP
AS
Arginine
Urea
NH3 + CO2
AcG
AcGP
AcGSA
AcORN
Putrescine
NCPUT
AGM
Polyamine Metabolism
(1)
(2)
(2')
(3)
(4)
(5)
(6)
(7)
(8)
(9)
(10)
(11)
(12)
(13)
(14)
(15)
(16)
(17)
(18)
(19)
(20)
(21)
(22)
(23)
(24)
(25)
(26)

Fig. 3

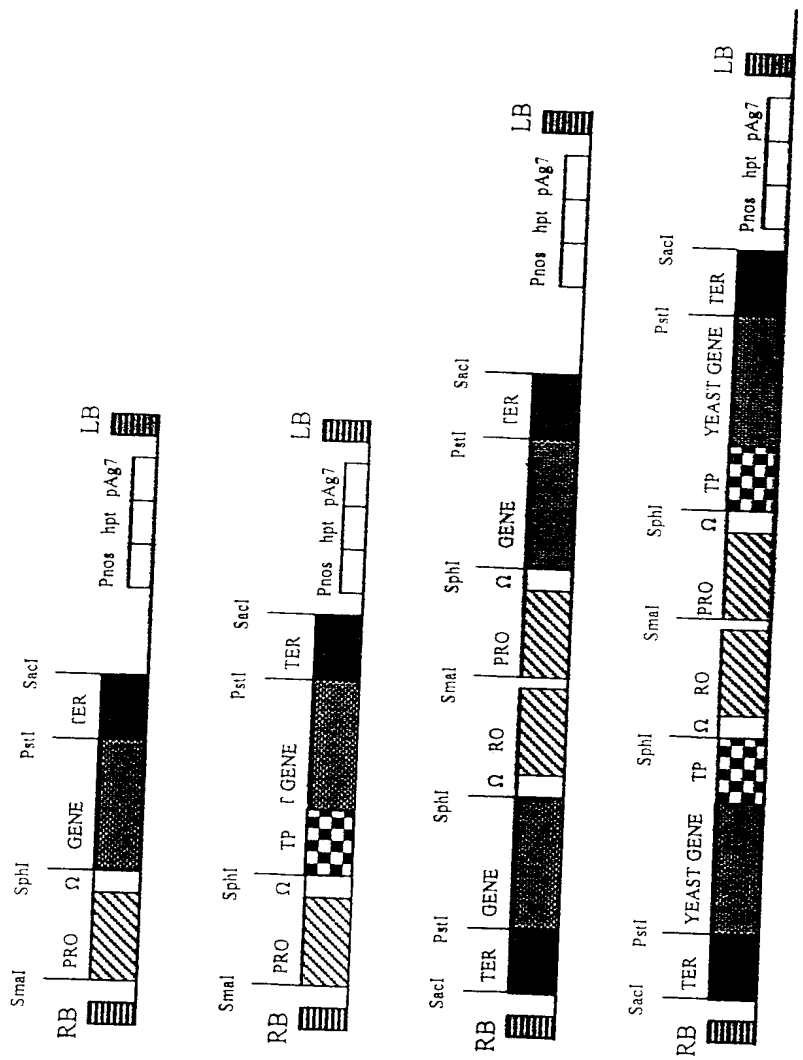
(a)
RB
SmaI
PRO
SphI
Ω
GENE
PstI
TER
SacI
Pnos hpt pAg7
LB
(b)
RB
SmaI
PRO
SphI
Ω
TP
GENE
PstI
TER
SacI
Pnos hpt pAg7
LB
(c)
RB
SacI
TER
PstI
GENE
SphI
Ω
RO
SmaI
PRO
SphI
Ω
GENE
PstI
TER
SacI
Pnos hpt pAg7
LB
(d)
RB
SacI
TER
PstI
YEAST GENE
SphI
TP
Ω
RO
SmaI
PRO
SphI
Ω
TP
YEAST GENE
PstI
TER
SacI
Pnos hpt pAg7
LB

Fig. 4

FIG. 5a- Met2

MSHTLKSKTLQELDIEEIKETNPLLKLVQGQRIVQVPELVLESGVVINNFPIAYKTWGTLNEAGDNVLVICHAL
TGSADVADWWGPLLGNDLAFDPSRFFIICLNSMGSPYGSFSPLTINEETGVRYGPEFPLCTVRDDVRAHRIVLD
SLGVKSIACVIGGSMGGMLSLEWAAMYGKEYVKNMVALATSARHSAWCISWSEAQRQSIYSDPNYLDGYYP
VEEQPVAGLSAARMSALLTYRTRNSFENKFSRRSPSIAQQQKAQREETRKPSTVSEHSLQIHNDGYKTKASTAI
AGISGQKGQSVVSTASSSDSLNSSTSMTSVSSVTGEVKDIKPAQTYFSAQSYLRYQGTKFINRFDANCYIAITRK
LDTHDLARDRVDDITEVLSTIQQPSLIIGIQSDGLFTYSEQEFLAEHIPKSQLEKIESPEATMPSYWSLS

FIG. 5b

ATGC ACTCATTTGA TAGTAAACTAAGTCATGTTA ATCGTTTGGA TTTGG CACACA
CCCACAAAT ATACACATTA CATATATATATATATTCAAA ATACAGCTGCGTCCAAT
AGA TGAGCTTCCG CTTCGTTGTA CAACCTACCTGCTATCTTGT TCACGGATAT TTC
TTGCTTT TAATAAACAA AAGTAACTCT AGAACAGTCA AGTCTTCGAT AATTTTTTT
AGTCACAGGGT CCGTCTAAAG TTTCTCTTTA-TTTGGAATAA TAGA AAAGA AAGAA
A AAAACGTAGTATAAA AGGAATGTCG CATACTTTAA AATCGAAAGCTCCAAGAG
CTGGACATTG AGGAGATTAA GGAAACTAAC CCATTGCTCA AACTAGTTCA AGGGC
AGAGG ATTGTTCAAG TTCCGGAACT AGTGCTTGAG TCTGGCGTGG TCATAAATAA
TTTCCCTATT GCTTATAAGA CGTGGGGTAC ACTGAATGAA GCTGGTGATAATGTTC
TGGTAATTTGTCAT GCCTTGACTG GGTCCGCAGA TGTTGCTGAC TGGTGGGGCC CT
CTTCTGGGTAACGACTTA GCATTCGACC CATCAAGGTT TTTTATCATA TGTTTAAACT
CTATGGGCTCTCCATATGGG TCTTTTTCGC CATTAACGAT AAATGAGGAG ACGGCGT
TAGATATGGACCCGAATTCCCA TTATGTACTG TGCGCGATGA CGTTAGAGCT CACA
GAATTG TTCTGGATTCTCTGGGAGTA AAGTCAATAG CCTGTGTTAT TGGTGGCTCT
ATGGGGGGGA TGCTGAGTTTGGAATGGGCT GCCATGTATG GTAAGGAATA TGTGA
AGAAT ATGGTTGCTC TGGCGACATCAGCAAGACAT TCTGCCTGGT GCATATCGTG
GTCTGAGGCT CAAAGACAAT CGATTTACTCAGATCCCAAC TACTTGGACG GGTAC T
ATCC GGTAGAGGAG CAACCTGTGG CCGGACTATCGGCTGCACGT ATGTCTGCAT TG
TTG ACGTA CAGGACAAGA AACAGTTTCG AGAACAAATTCTCCAGAAGA TCTCCTTC
AA TAGCACAACA ACAAAAAGCT CAAAGGGAGG AGACACGCAAACCATCTACT GTCA
GCGAAC ACTCCCTACA AATCCACAAT GATGGGTATA AAACAAAAGC CAGCACTGCC
ATCGCTGGCA TTTCTGGGCA AAAAGGTCAA AGCGTGGTGT CCACCGCATCTTCT TC
GGAT TCATTGAATT CTTCAACATC GATGACTTCG GTAAGTTCTG TAACGGGTGA AG
TG AAGGAC ATAAAGCCTG CGCAGACGTA TTTTTCTGCA CAAAGTTACT TGAGGTAC
CAGGGCACAAAG TTCATCAATA GGTTCGACGC CAATTGTTAC ATTGCCATCA CACG
TAAACTGGATACGCAC GATTTGGCAA GAGACAGAGT AGATGACATC ACTGAGGTCC
TTTCTACCATCCAACAACCA TCCCTGATCA TCGGTATCCA ATCTGATGGA CTGTTCAC
AT ATTCAGAACAAGAATTTTTG GCTGAGCACA TACCGAAGTC GCAATTAGAA AAAA
TTGAAT CTCCCGAAGCCACGATGCCT TCCTATTGGA GTTTAAGCTG A

FIG. 5c- Met25

MPSHFDTVQLHAGQENPGDNAHRSRAVPIYATTSYVFENSKHGSQLFGLEVPGYVYSRFQ
NPTSNVLEERIAALEGGAAALAVSSGQAAQTLAIQGLAHTGDNIVSTSYLYGGTYNQFKIS
FKRFGIEARFVEGDNPEEFEKVFDERTKAVYLETIGNPKYNVPDFEKIVAIAHKHGIPVVVD
NTFGAGGYFCQPIKYGADIVTHSATKWIGGHGTTIGGIIVDSGKFPWKDYPEKFPQFSQPAE
GYHGTIYNEAYGNLAYIVHVRTELLRDLGPLMNPFASFLLLQGVETLSLRAERHGENALK
LAKWLEQSPYVSWVSYPGLASHSHHENAKKYLSNGFGGVLSFGVKDLPNADKETDPFKL
SGAQVVDNLKLASNLANVGDAKTLVIAPYFTTHKQLNDKEKLASGVTKDLIRVSVGIEFID
DIIADFQQSFETVFAGQKP

FIG. 5d

ATG CCATCTCATT TCGATACTGT TCAACTACACGCCGGCCAAG AGAACCCTGG TGACAAT
GCT CACAGATCCA GAGCTGTACCAATTTACGCC ACCACTTCTT ATGTTTTCGA AAACTCTA
AG CATGGTTCGC AATTGTTTGG TCTAGAAGTTCCAGGTTACG TCTATTCCCG TTTCCAAAAC
CCAACCAGTA ATGTTTTGGA AGAAAGAATTGCTGCTTTAG AAGGTGGTGC TGCTGCTTTG
GCTGTTTCCT CCGGTCAAGC CGCTCAAACCCTTGCCATCC AAGGTTTGGC ACACACTGGT
GACAACATCG TTTCCACTTC TTACTTATACGGTGGTACTT ATAACCAGTT CAAAATCTCG
TTCAAAAGAT TTGGTATCGA GGCTAGATTT GTTGAAGGTG ACAATCCAGA AGAATTCGAA
AAGGTCTTTG ATGAAAGAAC CAAGGCTGTTTATTTGGAAA CCATTGGTAA TCCAAAGTAC
AATGTTCCGG ATTTTGAAAA AATTGTTGCA ATTGCTCACA AACACGGTAT TCCAGTTGTC
GTTGACAACA CATTTGGTGC CGGTGGTTACTTCTGTCAGC CAATTAAATA CGGTGCTGAT
ATTGTAACAC ATTCTGCTAC CAAATGGATTGGTGGTCATG GTACTACTAT CGGTGGTATT
ATTGTTGACT CTGGTAAGTT CCCATGGAAG GACTACCCAG AAAAGTTCCC TCAATTCTCT
CAACCTGCCG AAGGATATCA CGGTACTATCTACAATGAAG CCTACGGTAA CTTGGCATAC
ATCGTTCATG TTAGAACTGA ACTATTAAGAGATTTGGGTC CATTGATGAA CCCATTTGCC
TCTTTCTTGC TACTACAAGG TGTTGAAACATTATCTTTGA GAGCTGAAAG ACACGGTGAA
AATGCATTGA AGTTAGCCAA ATGGTTAGAACAATCCCCAT ACGTATCTTG GGTTTCATAC
CCTGGTTTAG CATCTCATTC TCATCATGAAAATGCTAAGA AGTATCTATC TAACGGTTTC
GGTGGTGTCT TATCTTTCGG TGTAAAAGACTTACCAAATG CCGACAAGGA AACTGACCCA
TTCAAACTTT CTGGTGCTCA AGTTGTTGACAATTTAAAGC TTGCCTCTAA CTTGGCCAAT
GTTGGTGATG CCAAGACCTT AGTCATTGCTCCATACTTCA CTACCCACAA ACAATTAAAT
GACAAAGAAA AGTTGGCATC TGGTGTTACC AAGGACTTAA TTCGTGTCTC TGTTGGTATC
GAATTTATTG ATGACATTAT TGCAGACTTCCAGCAATCTT TTGAAACTGT TTTCGCTGGC
CAAAAACCAT G

FIG. 5e- metX

MPTSEQNEFSHGSVGVVYTQSIRFESLTLEGGETITPLEIAYETYGTLNEKKDNAILVCHAL
SGDAHAAGFHEGDKRPGWWDYYIGPGKSFDTNRYFIISSNVIGGCKGSSGPLTINGKNGKP
FQSTFPFVSIGDMVNAQEKLISHFGIHKLFAVAGGSMGGMQALQWSVAYPDRLKNCIVMA
SSSEHSAQQIAFNEVGRQAILSDPNWNQGLYTQENRPSKGLALARMMGHITYLSDEMMRE
KFGRKPPKGNIQSTDFAVGSYLIYQGESFVDRFDANSYIYVTKALDHFSLGTGKELTKVLA
KVRCRFLVVAYTSDWLYPPYQSEEIVKSLEVNAVPVSFVELNNPAGRHDSFLLPSEQQDSI
LRDFLSSTDEGVFL

FIG. 5f

ATGCCTACC TCCGAACAGA ACGAGTTTTC CCACGGATCC GTAGGTGTCG TATATACTCA
GAGCATTCGA TTTGAGTCTT TGACTCTAGA GGGGGGTGAA ACCATCACTCTCTTGAAAT
TGCCTACGAA ACGTATGGCA CTCTCAATGA AAAAAAAGAC AATGCCATTC TAGTTTG
CCATGCGCTTTCG GGAGATGCTC ATGCAGCAGG TTTCCATGAA GGAGACAAAC GTCCT
GGCTGGTGGGATTAT TATATTGGAC CGGGCAAATC CTTTGATACC AATCGTTACT
TTATCATTTCTTCCAACGTA ATTGGTGGTT GTAAGGGTTC CAGTGGACCA CTTACCATCA
ATGGGAAAAATGGAAAACCA TTCCAATCCA CTTTTCCCTT TGTCTCCATA GGAGA TAT
GGTGAATGCTCAAGAAAAATTA ATCAGCCATT TTGGAATTCA TAAACTATTT GCTGT TGCCG
GTGGTTCGATGGGTGGAATG CAAGCCTTAC AATGGTCAGT CGCATACCCA GATCGGCTCA
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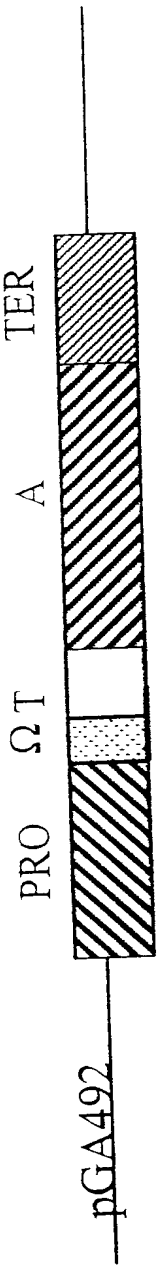


Fig. 6

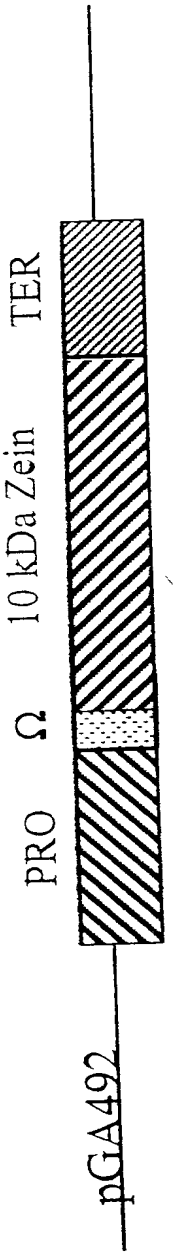


Fig. 7

Fig. 8a

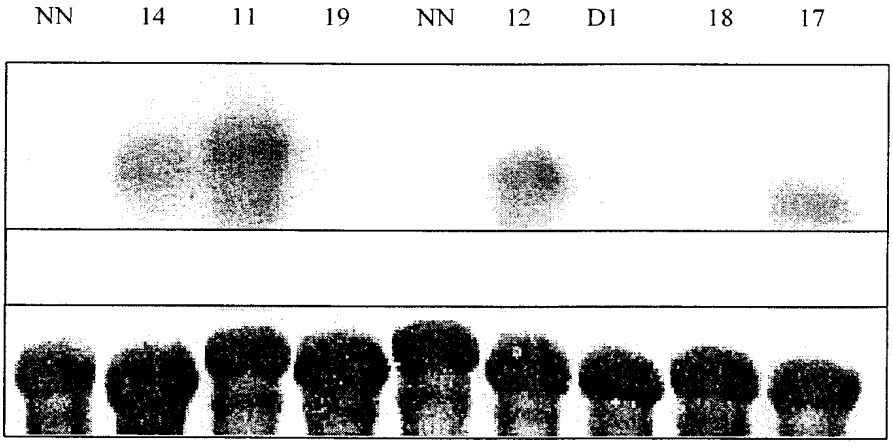
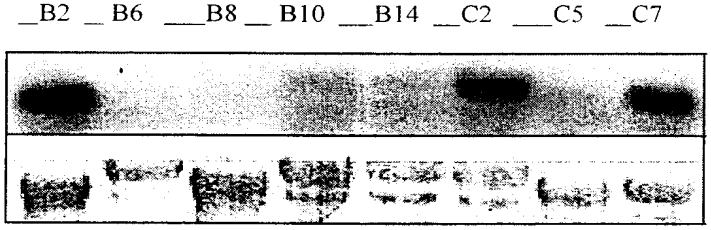


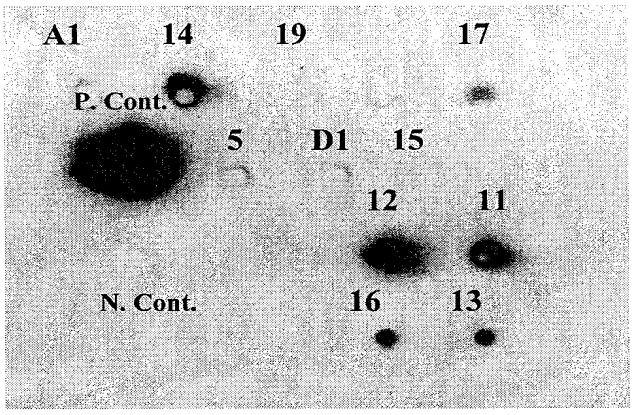
Fig. 8b



C - Cytozol

B - Chloroplast

Fig. 9a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19
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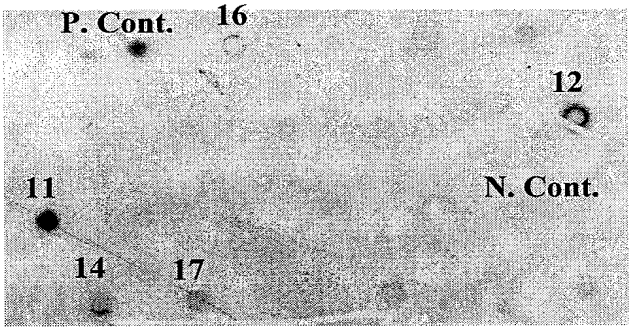
P. Cont.
5
D1
15
12
11
N. Cont.
16
13

Fig. 9b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N. Cont.
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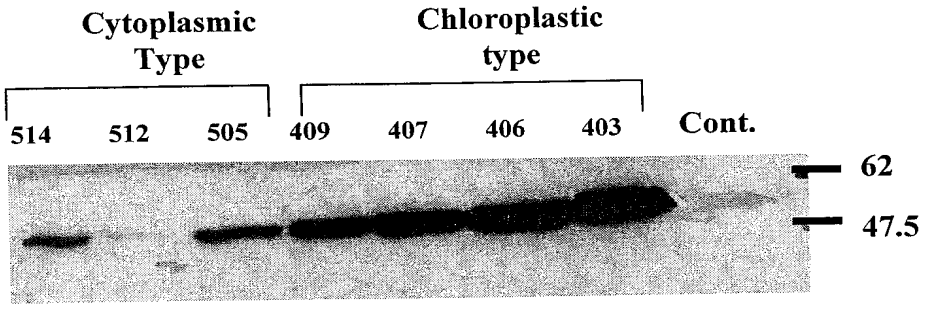
Fig. 10a



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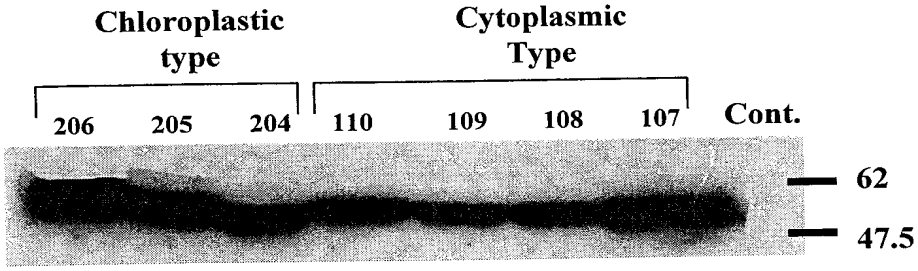


Fig. 11a

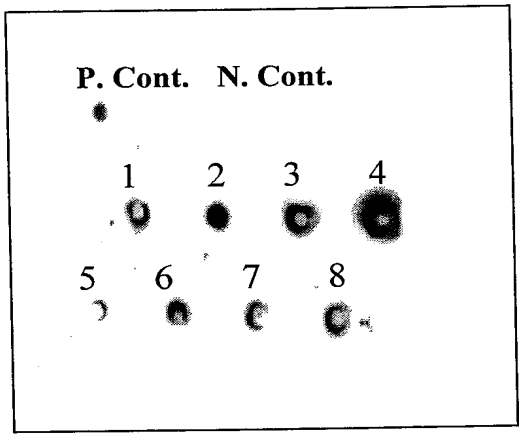


Fig. 11b

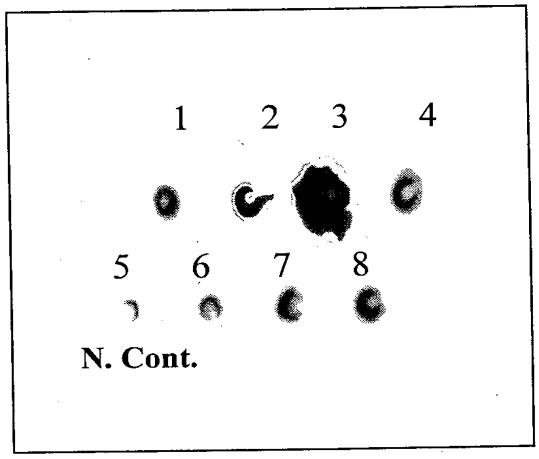


Fig 12a

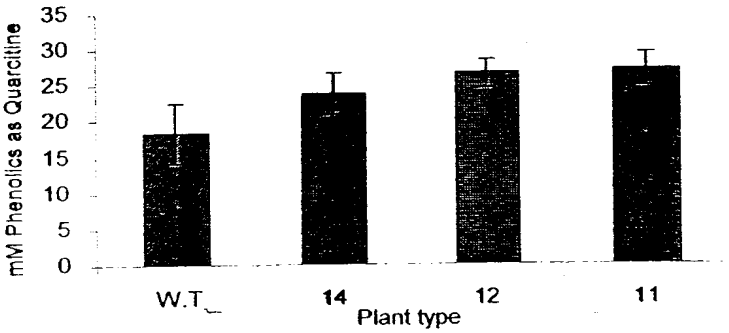


Fig.12b

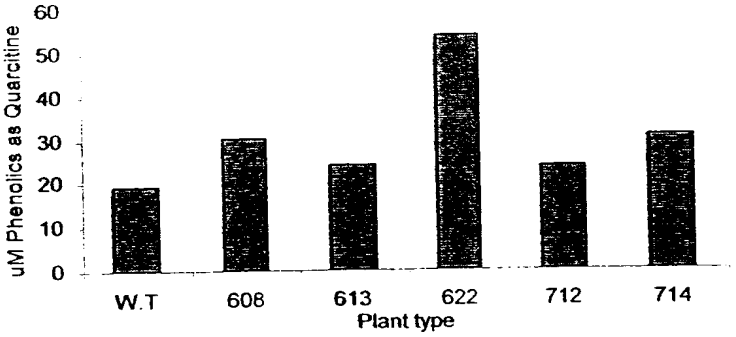


Fig. 13

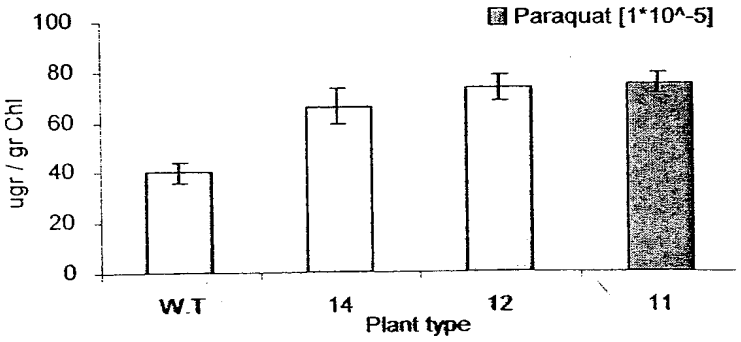
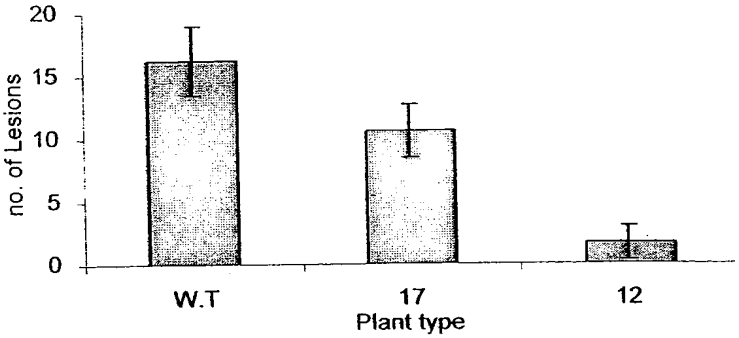


Fig. 14a



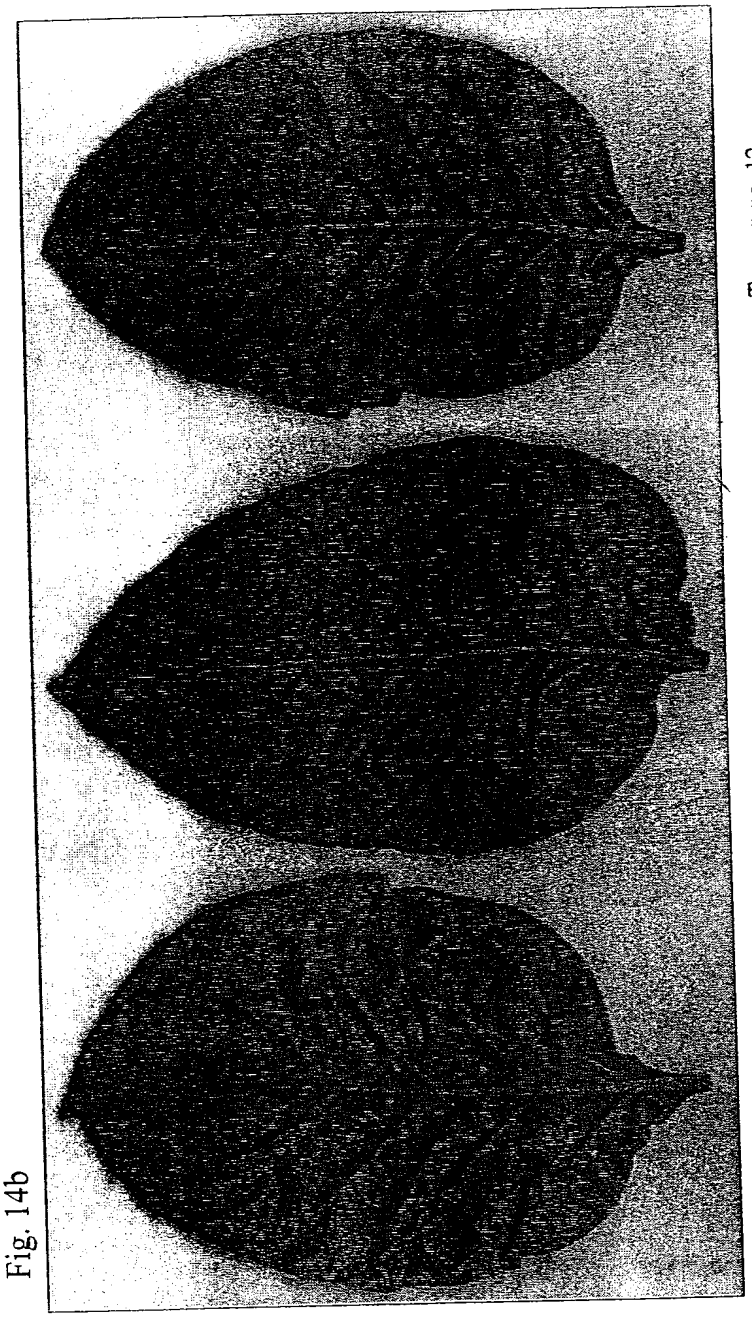
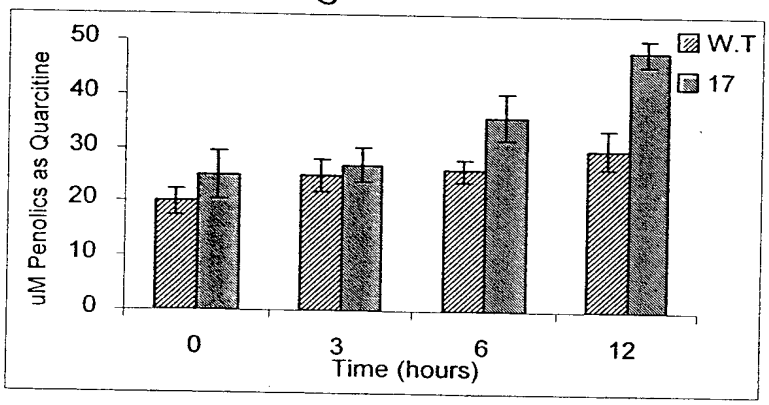


Fig. 14b

Fig. 15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40
30
20
10
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0
3
6
12
Time (hours)
W.T
17

Fig. 16

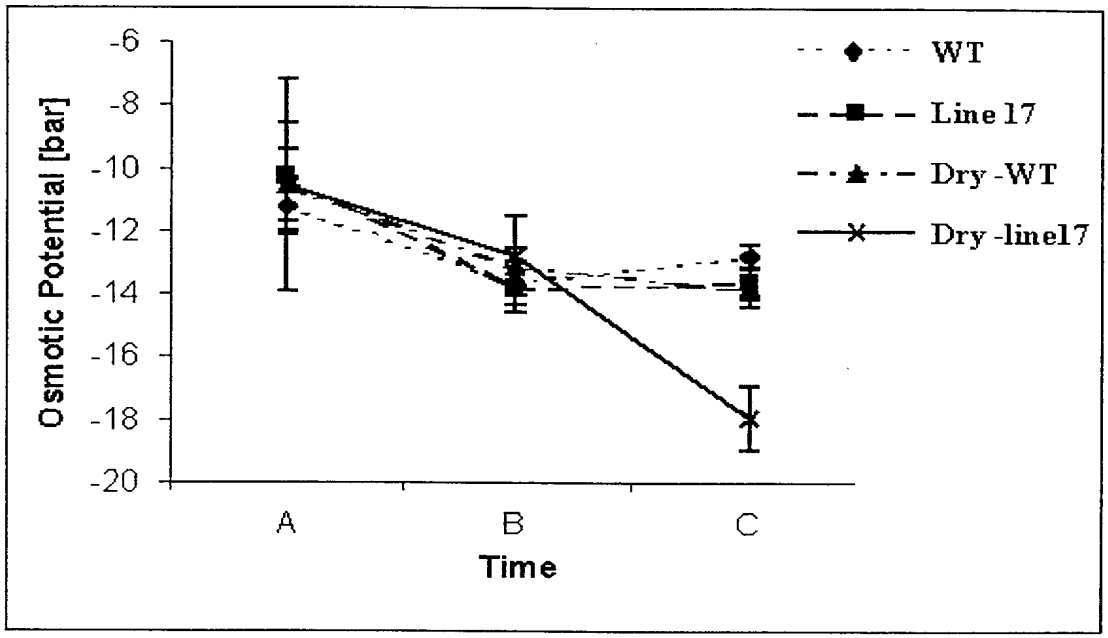
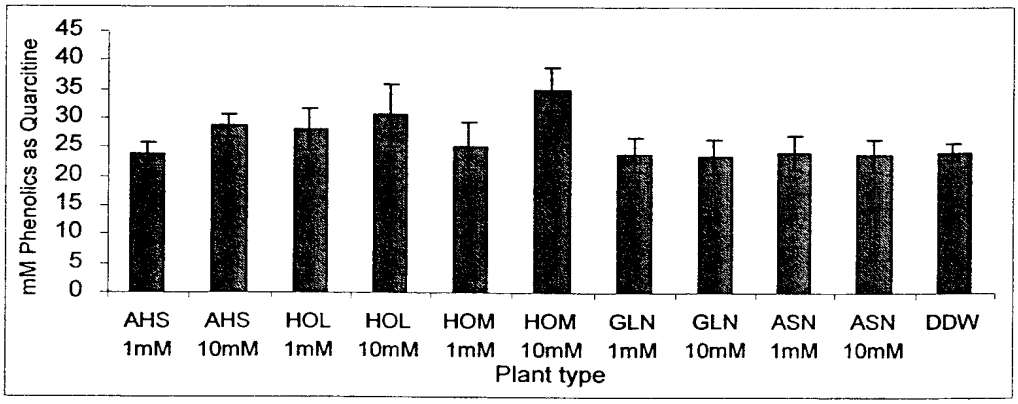

Osmotic Potential [bar]
-6
-8
-10
-12
-14
-16
-18
-20
A
B
C
Time
WT
Line 17
Dry -WT
Dry -line17

Fig. 17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40
35
30
25
20
15
10
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0
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AHS 10mM
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HOM 10mM
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GLN 10mM
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ASN 10mM
DDW
Plant type

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50 55 60

Asn Val Leu Glu Glu Arg Ile Ala Ala Leu Glu Gly Gly Ala Ala Ala
65 70 75 80

Leu Ala Val Ser Ser Gly Gln Ala Ala Gln Thr Leu Ala Ile Gln Gly
85 90 95

Leu Ala His Thr Gly Asp Asn Ile Val Ser Thr Ser Tyr Leu Tyr Gly
100 105 110

Gly Thr Tyr Asn Gln Phe Lys Ile Ser Phe Lys Arg Phe Gly Ile Glu
115 120 125

Ala Arg Phe Val Glu Gly Asp Asn Pro Glu Glu Phe Glu Lys Val Phe
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Thr Asp Phe Ala Val Gly Ser Tyr Leu Ile Tyr Gln Gly Glu Ser Phe
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Val Asp Arg Phe Asp Ala Asn Ser Tyr Ile Tyr Val Thr Lys Ala Leu
        275                 280                 285

Asp His Phe Ser Leu Gly Thr Gly Lys Glu Leu Thr Lys Val Leu Ala
    290                 295                 300

Lys Val Arg Cys Arg Phe Leu Val Val Ala Tyr Thr Ser Asp Trp Leu
305                 310                 315                 320

Tyr Pro Pro Tyr Gln Ser Glu Glu Ile Val Lys Ser Leu Glu Val Asn
                325                 330                 335

Ala Val Pro Val Ser Phe Val Glu Leu Asn Asn Pro Ala Gly Arg His
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Phe Leu Ser Ser Thr Asp Glu Gly Val Phe Leu
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